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BIOASSAY PROCEDURES FOR THE OCEAN DISPOSAL PERMIT PROGRAM

ENVIRONMENTAL RESEARCH LABORATORY

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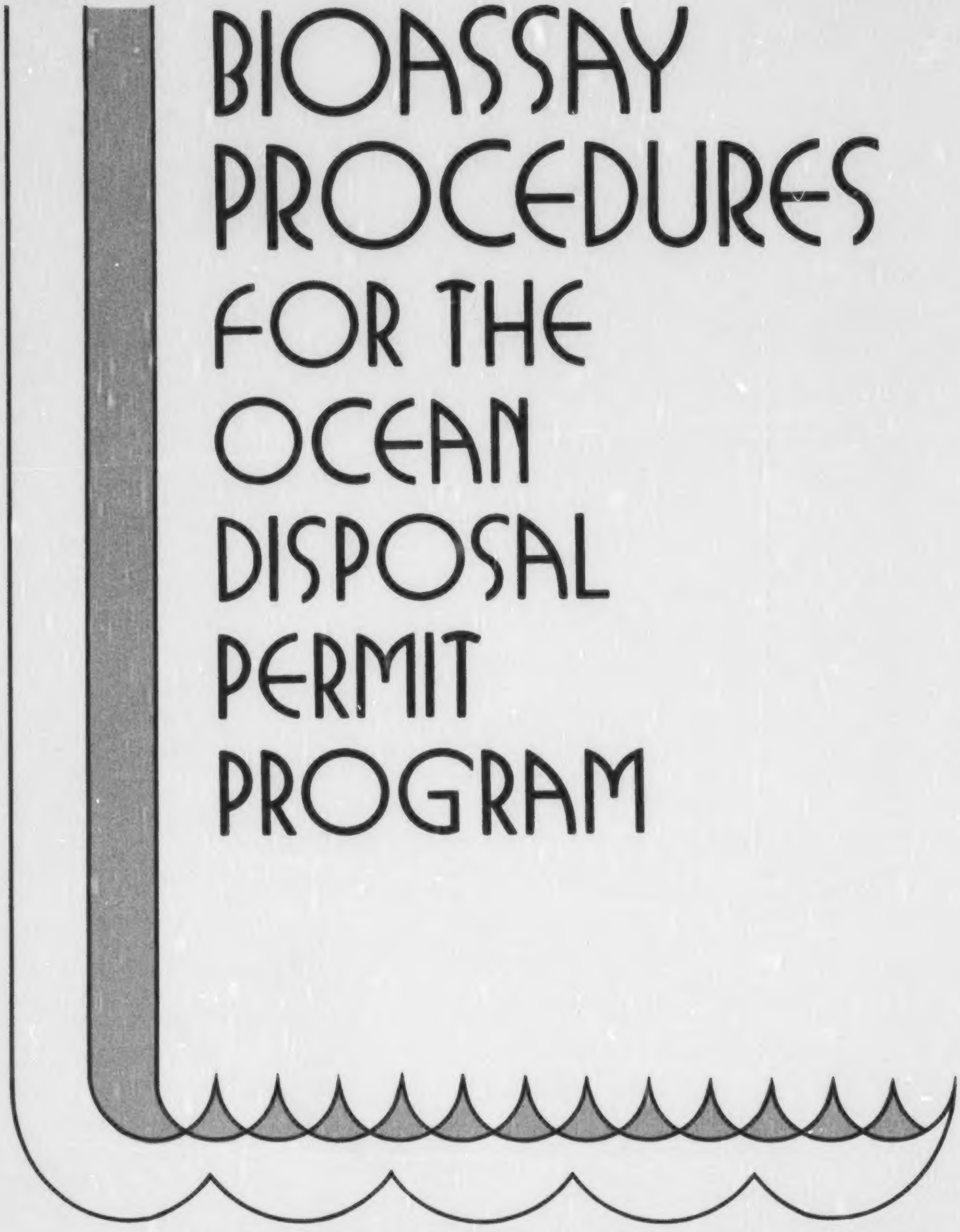

BIOASSAY PROCEDURES FOR THE OCEAN DISPOSAL PERMIT PROGRAM

ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
GULF BREEZE, FLORIDA 32561

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BIOASSAY PROCEDURES FOR THE OCEAN DISPOSAL PERMIT PROGRAM

by

Environmental Research Laboratory
Office of Research and Development
Gulf Breeze-Narragansett-Corvallis

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
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GULF BREEZE, FLORIDA 32561

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FOREWORD

Both the Marine Protection, Research, and Sanctuaries Act of 1972 (Sec. 102c) and the Federal Water Pollution Control Act Amendments of 1972 (Sec. 403c) require that applications for permits for the disposal of any materials into the ocean be evaluated on the basis of their ecological impact on the marine environment. The disposal of chemical or biological warfare agents and high-level radioactive wastes is prohibited. Organohalogen compounds, mercury, cadmium, and petroleum products cannot be ocean disposed except when present only as trace contaminants of waste materials. All other materials must meet the limiting permissible concentration (LPC) of the total pollutant before they may be disposed of in the marine environment.

The LPC is defined as that concentration of a waste material or chemical constituent in the receiving water which, after reasonable allowance for initial mixing in the mixing zone, will not exceed 0.01 of a concentration shown to be toxic to appropriate, sensitive marine organisms in a bioassay or otherwise shown to be detrimental to the marine environment. Therefore, the LPC concept and the bioassay results on which the LPC is calculated represent the essential technical criteria of the Ocean Disposal Permit Program.

Bioassay procedures described in this manual were developed for use by EPA personnel in carrying out the Ocean Disposal Permit Program. They are not intended as official EPA procedures or standard methods, but as guides to EPA ocean-dumping personnel when prescribing to permit applicants what bioassay tests are required. Under the regulations of the Ocean Disposal Permit Program, an EPA Regional Administrator has the discretionary authority to require the permit applicant's performance of any of these enclosed methods, modifications of these methods, or completely different methods. The Regional Administrator's decision regarding the requirement for the performance of bioassay tests depends to a great extent on the type and amount of waste, location of dump site, and proposed method of disposal as well as other technical considerations.

Dr. Thomas W. Duke
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ABSTRACT

The bioassay procedures given in this manual were developed to provide tests for conducting toxicity evaluations of waste materials considered for ocean disposal under EPA's Ocean Disposal Permit Program.

Nine bioassay procedures are described; three are considered "special" and are not recommended for routine use. The procedures specify the use of various organisms representing several trophic levels. Both flow-through and static tests are included. Methods given vary in their utility and complexity of performance. The procedures are not intended to be considered "standard methods," but, depending on the judgment of the EPA Regional Administrator responsible for the managing of the permit program, are to be used as reference methods or official methods.

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The bioassay procedures given in this manual are the result of the Ocean Dumping Bioassay Committee's deliberations and represent selection of methodology developed at various EPA laboratories. The laboratories and investigators involved are as follows:

Continuous-flow Method for Acute Toxicity Tests Using Fish and Macroinvertebrates

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SECTION I

INTRODUCTION

The bioassays procedures given in this manual were established to provide procedures for conducting biological evaluation of waste materials to be disposed of in the ocean. Tests conducted according to these bioassay procedures will provide information on the relative toxicity of various materials to be disposed. However, these bioassay procedures, like all laboratory bioassay methods, are attempts at simulation of actual conditions and therefore suffer all the inaccuracies inherent to simulation systems.

Although these bioassay procedures are not "standard" EPA methods, they are intended as guides for those involved in evaluating ocean dumping permits. Accordingly, each method differs in detail and style and does not conform to a standard format. Permit applicants are expected to modify bioassay procedures according to both the nature of the waste material and the type of procedure involved.

Three of the bioassay procedures presented in this manual have been classified as "special" (i.e., not to be used routinely). These procedures are the oyster shell growth procedure, the chronic fish egg-to-egg procedure, and the acetylcholinesterase inhibition test. The remaining bioassays lend themselves to more routine measurement.

The Ocean Dumping Bioassay Committee stated that the minimum number of species to be used in an evaluation of a dumping permit should be three. These species should be selected from the different taxonomic groups listed in the section on the continuous-flow method for acute toxicity tests using fish and macroinvertebrates (see page 71). Note that the brine shrimp (Artemia salina) is not on this list. The committee felt that the brine shrimp was not a satisfactory organism for use in marine bioassays. However, they are readily available, and tests can easily be conducted to compare their sensitivity for a selected waste material to that of indigenous species from a particular dump zone. We recommended that indigenous organisms be used whenever possible in addition to those organisms recommended in this manual.

The EPA bioassay working group intends to periodically revise these bioassay procedures as new information becomes available. An annual revision is anticipated.

SECTION II

BIOASSAY PROCEDURES FOR ROUTINE APPLICATION

A. BACKGROUND INFORMATION FOR THE PERFORMANCE OF PHYTOPLANKTON MARINE BIOASSAYS

The primary producer populations of estuaries consist principally of microscopic phytoplankton. In their role of storing potential energy, via photosynthesis, these organisms represent the primary energy input into aquatic ecosystems (Joint Industry/Government, 1969). For this reason, it is imperative that water quality conditions be favorable to their growth and reproduction if serious alterations in other components of marine communities are to be avoided.

Under natural conditions, both the qualitative and quantitative aspects of phytoplankton population dynamics display a high degree of seasonability, characterized by well-defined succession patterns. It is essential that not only the productivity of various systems be maintained, but also the relative abundance of species according to normal seasonal compositions; as primary herbivore populations exhibit selectivity in their grazing patterns. Consequently, while a pollutant may seem to have no apparent adverse effect on the total primary production, it may have drastically altered community structure and composition. Such alterations often occur when sensitive indigenous species are eliminated and ecologically less desirable, but other equally photosynthetically active species dominate. If the more resistant species is incompatible with the feeding and/or nutritional requirements of primary herbivore populations, then energy transfer to higher trophic levels will be affected and contribute ultimately to significant effects on naturally occurring desirable populations. To adequately describe and predict the potential effects of a toxicant upon an estuarine ecosystem response, data for the phytoplankton is a necessity.

1. Species Selection

In the design of a bioassay program, the selection of test species is pivotal to the acquisition of realistic and meaningful information. Algal culture techniques historically have focused upon developing suitable culture media to sustain complete life cycles. Nutritional levels and medium composition often bore little resemblance to the actual environmental conditions the organism encountered. Furthermore, research was often limited to a few species that were readily maintained in the laboratory.

Within the last decade, culture techniques have greatly broadened the scope of species available for investigation.

In choosing species for bioassays, the following criteria are useful guides:

a. Whenever possible, indigenous species representing a diversity of phylogenetic types from the major seasonal successions should be studied.

b. Since sensitivities vary among species, when possible, select the more sensitive species for bioassay.

c. From seasonal and laboratory studies, conditions of greatest vulnerabilities should also be identified for the species selected.

d. Since a bioassay basically measures the response of an organism to the product of toxicant concentration and exposure time, the rate of response of the test species must be considered. Both test species and culture conditions should permit growth rates of 0.5-1.0 doublings per day under non-stress conditions.

The above criteria offer maximum flexibility for the experienced researcher. For workers with limited funds and expertise, two species are recommended, both to be used if indigenous forms are unavailable. It is also recommended that these species be used in conjunction with others to serve as controls on the systems being tested. Skeletonema costatum is an ecologically important phytoplankter that is common to a wide geographic range of neretic waters. Thalassiosira pseudonana, while of lesser ecological significance, is sensitive to heavy metals and has an 8-hour generation time which offers greater practical value in the establishment of toxicological responses.

2. Culture Conditions

The culture conditions for the test species generally should reflect their natural conditions. In order to develop some semblance of uniformity, two basic regimes are recommended. For temperate species, a temperature of 20° ± 2°C, light intensity of 2500-5000 lux on a 14-hour light and 10-hour dark cycle (14:10 cycle) is desirable. For cold water forms, a temperature of 8° ± 2°C, 2500-500 lux on a 10:14 cycle is recomended. Stock cultures of the test species are to be maintained in enriched natural (Table 1-A), or synthetic sea waters (Table 2-A).

They should be transferred to the nutritionally dilute culture medium and allowed to go through two complete growth cycles prior to use in a bioassay. This is necessary since nutritional history can have marked effects upon responses. We have found up to five fold differences in responses of bioassay organisms maintained under high and natural nutrient levels (Gentile et al. 1973). Stock cultures should be maintained bacteria-free whenever possible and transferred at 1-2 week intervals.

TABLE 1-A. SEA WATER AND STERILITY ENRICHMENTS

Sea water enrichments for stock algal culture maintenance (After Guillard and Ryther, 1962):

$NaNO_3$	75 mg/liter
$NaH_2PO_4.H_2O$	5 mg/ℓ
$Na_2SiO_3.9H_2O$	10 mg/ℓ
Vitamins:	
Thiamine HCl	0.10 mg/ℓ
Biotin	0.50 μg/ℓ
B_{12}	0.50 μg/ℓ
Trace metals:	
$CuSO_4.5H_2O$	0.002 mg/ℓ
$ZnSO_45H_2O$	0.004 mg/ℓ
$CoCl_2.6H_2O$	0.002 mg/ℓ
$MnCl_2.4H_2O$	0.036 mg/ℓ
$NaMoO_4.2H_2O$	0.001 mg/ℓ
Fe-sequestrine	1.0 mg (0.13 mg Fe)/ℓ

Buffer:

TRIS-500 mg/ℓ @ pH 7.8-8.2

Sterility enrichments to be added to enriched sea water medium above before autoclaving:

Sodium glutamate	250 mg/ℓ
Sodium acetate	250 mg/ℓ
Cycline	250 mg/ℓ
Nutrient agar	50 mg/ℓ
Sucrose	250 mg/ℓ
Sodium lactate	250 mg/ℓ
L & D alanine	250 mg/ℓ

TABLE 2-A. SYNTHETIC SEA WATER FORMULATION FOR ALGAL ASSAYS (After Kester et al., 1967).

Compound	Concentration/liter
NaCl	24.00 g
Na_2SO_4	4.00 g
H_3BO_3	0.03 g
$CaCl_2 . 2H_2O$	1.47 g
$MgCl_2 . 6H_2O$	10.78 g
$Na_2Si:O_3.9H_2O$ *	30.00 mg
KCl	700.00 mg
$NaHCO_3$	200.00 mg

*Prepare stock solution in deionized water and adjust to pH 7.8-8.2

3. Sea Water

The choice of sea water is dictated by availability, quality and cost. Natural sea water can often be used for bioassays even though inherent variabilities in quality may complicate analysis of results. Clean offshore water is suitable if proper precautions during collection and processing are observed. In general, synthetic sea water is preferred for the constancy of its composition and quality even though trace contaminants must be removed by additional purification. The cost of the required chemicals and purification are usually equivalent to the expense of collecting, transporting and processing natural sea water.

a. Natural Sea Water

Sea water is collected from 3-10 meters (to avoid surface contamination) with a non-metallic water sampler, and transported in autoclavable polyethylene carboys. Glass is also suitable if breakage can be prevented. Within 24 hours, the water is filtered through acid-washed membrane filters in a non-metallic filtration system. Filtered sea water is then stored at 4°C in the dark.

b. Synthetic Sea Water

A modified synthetic sea water formulation (Table 2-A) has been developed from Kester et al. (1967). This sea water is recommended for fish, invertebrates and plankton bioassays. This synthetic sea water has been endorsed by the Environmental Protection Agency, the 14th Edition of Standard Methods and the A.S.T.M. Committee on Bioassays.

c. Salinity

A salinity of 30 $^{o}/_{oo}$ is recommended for all bioassays. Salinity adjustments on natural or synthetic sea waters should be made with glass distilled or deionized water.

d. Sterilization

Sterilization of stock culture maintenance medium can be satisfactorily achieved by autoclaving since the pH is stabilized by the presence of TRIS-buffer. Since bioassay medium cannot be autoclaved, two alternative methods are recommended: 1) positive pressure filtration and/or 2) pasteurization (60° ± 2° C for 4 hours). These treatments will not appreciably alter the physical-chemical properties of the sea water but will provide effective sterilization.

The medium should, however, be filtered (0.45μ) through a previously acid washed filter 2 N HCl). Removal of residual acid is accomplished by rinsing the filter with distilled/deionized water and discarding the first liter of filtered sea water. Medium should be stored in acid stripped borosilicate glass or linear polyethylene carboys. To these can be connected a sterile dispensing tower for distributing media.

Sterility checks are made weekly on this test medium by inoculating 2 ml aliquot of sea water into 10 ml of sterile water enriched as in Table 3-A. The tubes are incubated at 20°C in the dark for up to one week. Contamination is indicated by turbidity and opalescence of the medium.

TABLE 3-A. NUTRIENT ENRICHMENTS FOR ALGAL BIOASSAY MEDIUM

Nutrient	Amount
$Na\ NO_3$	4.42 mg/ℓ (50 μMN)
K_2HPO_4	0.87 mg/ℓ 5 μMP)
Thiamine	100.00 μg/ℓ
Biotin	0.50 μg/ℓ
B_{12}	0.50 μg/ℓ
Fe*	25.00 μg/ℓ
Mn	10.00 μg/ℓ
Zn	1.00 μg/ℓ
Mo	0.50 μg/ℓ
Co	0.10 μg/ℓ
Cu	0.10 μg/ℓ

*Fe as Cl: Dissolve iron sponge or filings in minimum HCl with warming and dilute to volume with deionized water.

4. Glassware

All glassware is high grade borosilicate glass (Pyrex/Kimax). The bioassays are performed in 125 ml Erlenmyer flasks, containing 50 ml of medium, and foam plugged. Glassware is dry-heat sterilized (170°C for 2 hours) rather than autoclaved, since the stem often carries metal contaminants which can interfere with bioassays involving metal toxicity.

Rigorous cleaning is necessary for all glassware to insure against contamination. Glassware is soaked in detergent, hand or mechanically brushed, rinsed in deionized water, totally immersed in 10% HNO_3 for 2-6 hours, thoroughly rinsed in double glass distilled or deionized water and air or oven dried.

For work involving the toxicity of metals, the glassware should receive the following post-wash treatment. To eliminate the problems of either positive or negative contamination, a monolayer of silico-polymer is applied to all surfaces contacting the sea water. Commercially available SC-87* is prepared as 5% solution in cyclohexane, poured into and drained, leaving a film on the surfaces of the glassware that have come in contact with the solution. The glassware is then air dried and oven cured at 150-175°C for 4 hours. The result is a completely non-wetable surface which, after a double glass distilled water rinse, is ready for use. One coating often lasts two or three assays before recoating is necessary. Recoating can be done over the old coating or a strong alkali (2N NAOH + 10% ETOH) can completely strip the old coating prior to recoating. In most instances, alcoholic-alkali stripping can be avoided by using hot detergent each time prior to recoating.

5. Bioassay Protocol

The bioassay design consists of three major integrated components: preparation of log-phase inoculum, nutrient enriched bioassay medium and toxicant solutions.

a. Inoculum

Inoculum for the bioassay is prepared by inoculating 0.5 ml (0.1-1.0 ml) of stock culture into triplicate 125 ml flasks containing nutrient enriched sea water at bioassay level (Table 3-A). At the point of inflection of the growth curve, inoculate three new flasks from this series and follow the second growth curve. Cells from this second or later transfers are suitable for use in the bioassay. These cells have now become adapted to the more natural nutrient levels, and their response will more closely reflect that expected from a natural population of the test species.

b. Bioassay Medium

Filter sterilized and/or pasteurized, enriched sea water is dispensed into a presterilized 1-2 liter flask that is compatible with a 50 ml Ace-dispenser (Cat. no. 8004, Ace Glass Co., Vineland, N.J.). Nutrients (Table 3-A) are aseptically added and inoculum (as described above) is added to give an initial cell density of 2,500 cells/ml to 10,000 cells/ml. Inoculation of the total medium volume permits the dispensing of a uniform cell population to all flasks. Initial cell density or biomass is measured. Fifty milliliters of enriched inoculated medium is dispensed into 125 ml flasks using a 50 ml Ace-dispenser in a sterile hood.

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*SC-87. Product of General Electric: distributed by Pierce Chemical Co., Rockford, Illinois.

The selection of an initial cell density will be dependent upon the sensitivity of the biomass parameter measuring system. For example, in clean systems using particle counters, initial cell densities of 2.5 x 10^3 microscopic counts are employed, initial cell densities of 1 x 10^4cells/ml may be appropriate. For extractive (ATP, Chl "a") or isotope techniques, the initial cell density can be kept low since the aliquot examined can be adjusted.

c. Toxicant

Toxicant solutions are prepared in distilled water or suitable solvent for hydrophobic compounds. Stock solutions or dilutions of a waste should be prepared to insure that the same volume is added at all test levels. This addition should not exceed one milliliter/50 ml of test medium. When working with waste effluents, a maximum of 5 ml addition is allowed since this will constitute a 10% maximum alteration in salinity. Toxicant additions are made to the flasks containing inoculated enriched sea water and placed in an incubator.

d. Design

The bioassay design is in part determined by the type of toxicant tested. A general format will include a screening of a broad range of concentrations from which levels are selected for a definitive evaluation. Generally, preliminary screenings should cover concentrations at four orders of magnitude with duplicate cultures at each level. The definitive assay should include one concentration above and two below the calculated 50% inhibition level using logarithmic bisection of intervals. Triplicate cultures should be used for the definitive bioassay.

Parameter measurement should be evaluated at least once every 24 hours for the duration of the experiment. This permits calculation of rates of response which are important in interpreting the behavior of the toxicant. The duration of the experiment should be adequate for the control population to complete its logarithmic growth phase and reach a stationary growth rate. It is also desirable to determine for the inhibited cultures: the duration of the lag-phase, maximum rate of growth and maximum yield (Figure 1-A). However, not all this information may be readily available from a single assay and all concentrations.

e. Modifications

The assay system described above uses small volumes (50 ml/125). This is not meant to frustrate the expansion of assay volumes. The systems can be easily scaled up to the following dimensions of 125/250; 250/500; 500/1,000. With larger volume systems media, dispensing can be made directly into the sterile flasks. Nutrients and test species can also be added to each flask. This increases the potential for variability and contamination but, with experience, difficulties may be minimized. The larger systems require more assay medium and space, however, greater volumes will permit more frequent analysis of a greater number of parameters. This

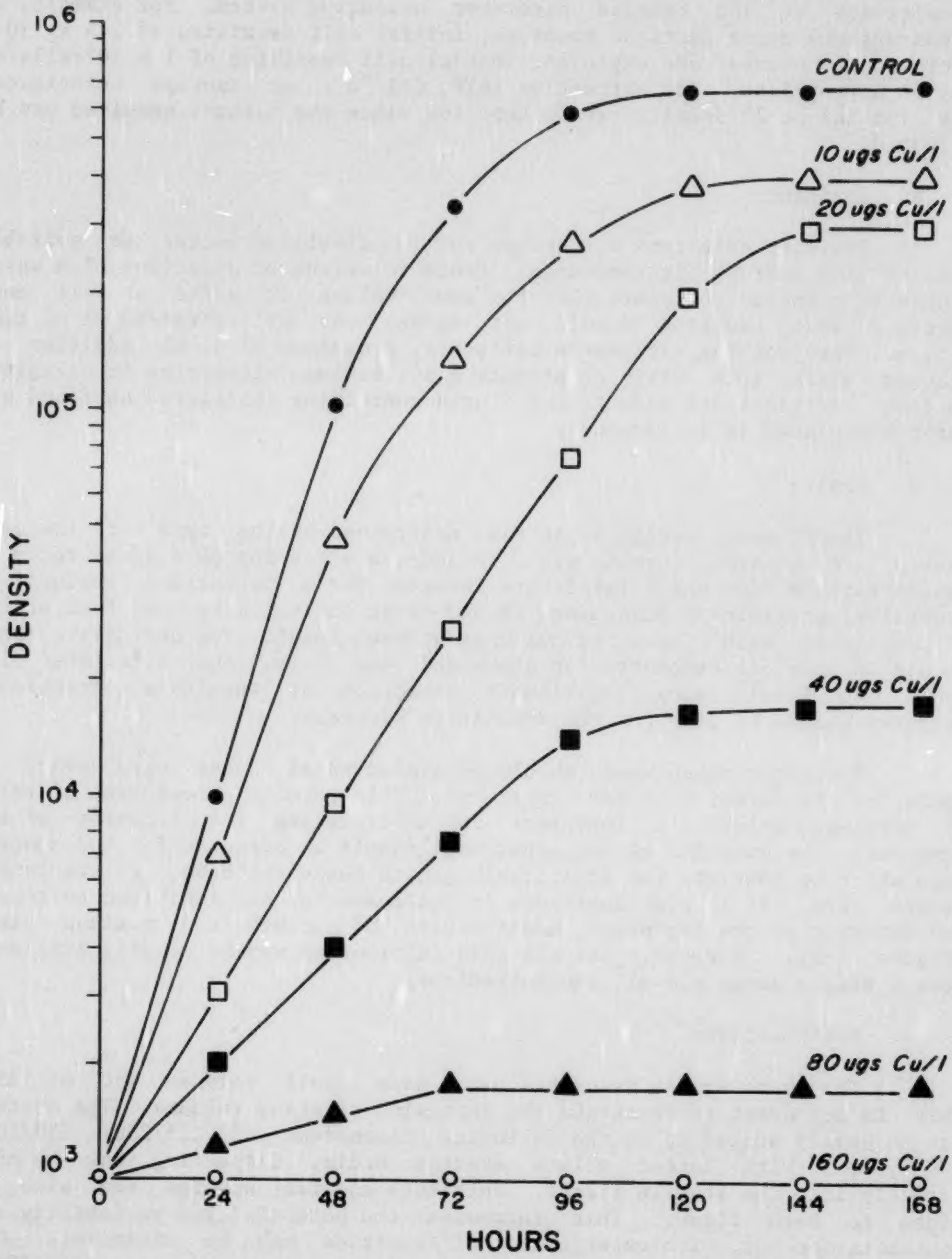

Figure 1-A. Hypothetical relationship between algal growth and toxicant concentration.

allows a more precise characterization of the anomalies resulting from specific pollutant exposures.

6. Parameters

There are a variety of parameters available that characterize the response of the algal cultures. These parameters are measures or indices of biomass at the time of sampling, which, when plotted against time, produce a growth response curve. This curve can be used to determine log-phase, rate of log-growth, and a maximum population density for control and exposed cultures.

a. Population Density

Microscopic measurements of cell density can be made using a haemocytometer, Palmer-Maloney Chamber, or inverted microscope with settling chambers. Details of these counting methods are available in the literature (Schwoerbel, 1970; Weber, 1973).

The microscopic methods present two problems: they are time-consuming when done properly and their statistical significance decrease significantly at cell densities below 1 x 10^4. Consequently, when large numbers of assays and replicates are required, it becomes impractical to count each assay microspically.

The most rapid, practical and statistically accurate measure of population density is with an electronic particle counter. The initial cost, while high, is offset by the increased work volume, accuracy and saving of time.

b. Population Biomass

Biomass values can be calculated from population density data by using cell dimensions and assuming the cell is a particular geometrical shape (i.e., sphere, cylinder, etc.). This method, being dependent on cell counts, is subject to the same limitations mentioned above.

Electronic particle counters can also give volumetric measurements, but usually such capabilities are obtained at additional cost. Still it is worth the expense, if large numbers of assays are anticipated.

c. Chlorophyl

Chlorphyl "a" is often used as a measure of algal biomass. Both spectro-photometric absorbance and fluorescence (*in vivo* and *in vitro*) techniques are available (Strickland and Parsons, 1968). The spectrophotometric technique lacks the sensitivity particularly at low cell densities. The fluorescent systems, however, are more sensitive and can be used at cell densities of less than 1 x 10^4 cells/ml. The *in vivo* fluorescent technique is particularly useful because it does not require extraction and is very sensitive.

A potential limitation of this measurement is the general variability of cellular chlorophyl "a" as a function of nutrition and environmental variables (Odum et al. 1959; Yentsch and Ryther, 1957; Yentsch and Menzel, 1963).

d. Carbon-14 Assimilation

Productivity measurements, based upon radioactive carbon assimilation, is a standard technique applicable to both fresh water and marine algae (Steeman-Neilson, 1952; McAllister, 1961; Jitts, 1963; Jenkins, 1965; Strickland and Parsons, 1968). This is usually used as a short term measure of photosynthetic activity. Culture aliquots may thus be pulse-labeled for four hours and to record C-14 label incormay thus be pulse-labeled for four hours and to record C-14 label incorporated by cells and use this relative value as a biomass index. This latter approach has shown a correlation to growth rates as measured by changes in cell number or biomass. Transient changes in C-14 assimilation, not reflecting long-term growth responses, have also been noted and warrant cautious interpretation of this data.

Adequate C-14 counting procedures may be obtained in Brandsom (1970) and Chase and Rabinowitz (1967).

e. ATP-Concentration

ATP has been suggested as a sensitive and accurate measure of living biomass due to a constancy of cellular ATP/carbon ratio (Holm-Hansen and Booth, 1966; Hamilton, Holm-Hansen, 1967; Holm-Hansen, 1969). Studies have demonstrated excellent correlation between ATP and direct measures of biomass (particle counting) and pulse labeling with carbon-14 (Gentile et al. 1973; Cheer et al. 1974). This technique requires instrumentation (about $5,000) and costs about $1.00 per analysis. Being a measure of living material, highly contaminated wastes (i.e., sludge) could provide excessive interference.

The above techniques all offer certain advantages or disadvantages, depending on the bioassay design, type of effluent tested, facilities and personnel.

Automated particle counting, while offering the most rapid, sensitive and statistically valid method, has limitations. The most restrictive is that related to particle interferences. The test compound or effluent must have low background in the particle size range of the test species or inevitable masking and errors will result. This limits the types of effluents to be evaluated by this technique, unless the particulate fraction can be removed without jeopardizing the toxic characteristics of the material.

The other methods work well in systems containing particulate material, but both chlorophyl "a" and carbon-uptake have potentially undesirable response patterns that can make data interpretation difficult.

ATP, on the other hand, appears to be an excellent indicator of living biomass though it is somewhat expensive to measure routinely and may not be appropriate for biologically contaminated wastes (i.e., sludge).

All data can be converted to percent control for any finite exposure period and the percent response plotted versus toxicant concentration (Figure 2-A). From this graph, the relationship between toxicant concentration and degree of inhibition can be determined.

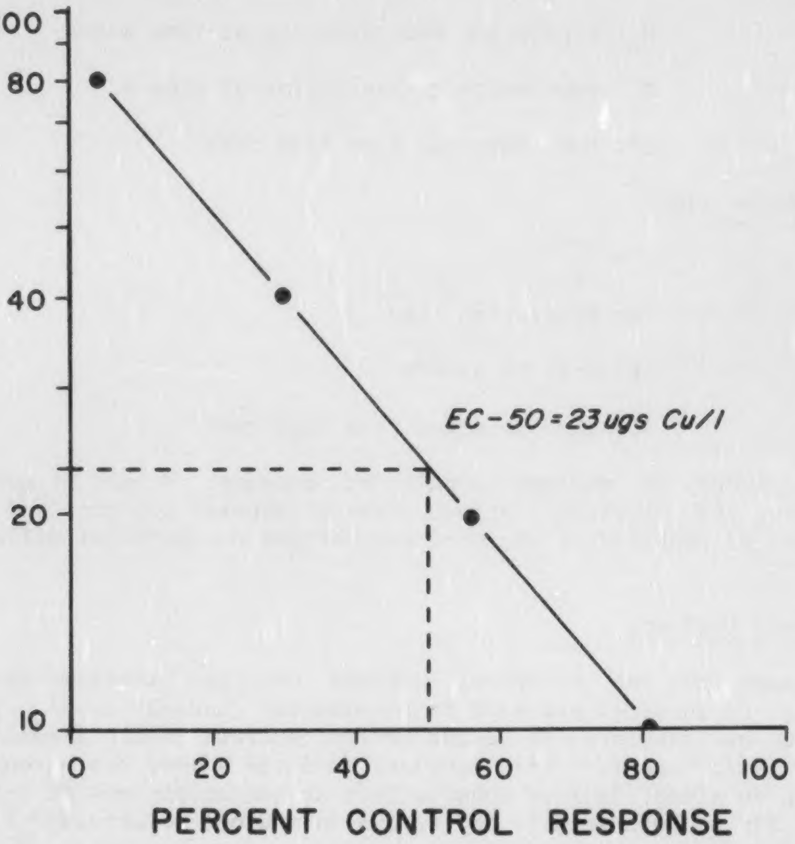

Figure 2-A. Relationship between percent of control growth rate (0-48 hrs.) and copper.

7. Data Presentation

The design of the bioassay requires a minimum of one observation every twenty-four hours for the duration of the experiment. Within this schedule, various options are available to the researcher. The basic data output represents a growth curve for all concentration examined. This may provide rate of growth:

$$k = \ell n \frac{N+}{N_o} / \Delta T$$

k: rate of growth

N_o: population concentration at time zero

N_t: population concentration at time t

ΔT: time interval from time zero

and generation time:

$$G = \frac{\Delta T}{K}$$

G: generation time

k: rate of growth

ΔT: time interval from time zero

and comparisons at maximum population density. Slopes of growth curves representing the logarithmic growth phase of exposed cultures and population biomass may be compared by standard statistical analysis for difference with controls.

8. Standard Toxicant

To insure that the technical aspects of the bioassay are properly performed, an internal standard is recommended (LaRoche et al. 1970). The compound we use routinely is sodium dedecyl sulfate (SDS), a surfactant and membrane lytic agent. This compound produces a very sharp response curve indicating an almost "all or none" effect at concentrations of 1-2 mg/ℓ. In addition, SDS is both soluble and stable in aqueous solutions.

While the use of an internal standard can serve as a quality assurance monitor, it does not, in itself, validate an experiment. There can be situations where the EC50 concentration for the standard toxicants in two experiments are essentially identical, but the control growth rates differ by a factor of two. The deviation of control growth from normal is an indication of a problem and this alone warrants the repetition of the experiment.

9. Applications

Algal bioassays, with their sensitivty and rapid response, are useful in many areas of water quality research.

a. The simplest application is for routine screening of potential toxicants. This represents a well-defined and controlled system where particle counting is recommended since interferences can usually be minimized. These studies should be designed to produce complete growth curves with both growth rate and maximum density output.

b. Another application of the algal bioassay is as an evaluation of water quality. If an impacted area is being investigated, water samples can be collected along a transect or matrix, depending on hydrographic data. The water is collected and processed according to techniques described in Section 4, and then inoculated with the test species that has been cultured in enriched water from a control station. Growth rate and population density can then be compared from station to station.

c. The algal assay can also be used to measure the biological impact of mixed effluents containing suspended solids. In this case, particle counting may not be practicable due to high levels of interference. Consequently, the growth of the algal culture can be monitored by obtaining daily aliquots and evaluating the ATP, chlorophyl "a", or measuring C-14 incorporation after pulse labeling the aliquot (2-4 hours) with NaH^{14} CO$_3$. The resulting data, when plotted semi-logarithmically with time, will produce a growth response curve that may be submitted to the interpretation discussed herein.

d. Mention must be made of *in situ* applications of phytoplankton bioassay. Using ATP, C-14 uptake and chlorophyl "a", both *in situ* living biomass and productivity of a water mass may be estimated. These studies can be made at the site, the samples preserved and analyzed at a later date. Such applications as evaluation of power plant entrainment and point source pollution monitoring, commonly use this approach.

10. Remarks

It should be stressed that important advances have been made by the utilization of phytoplankton bioassays in the establishment of realistic water quality criteria for marine life.

Fundamental biological anomalies in these organisms could impair survival of high trophic levels and certainly be associated with specific pollutant exposures. However, it should be noted that problems exist in the application of laboratory findings to conditions which may be found in the natural environment. One scientific discipline which has greatly been neglected in this area is certainly that of phytoplankton systematics. As a consequence, it is felt that, in many instances of *in situ* evaluation of phytoplankton productivity, identification of species will reveal the importance of knowing the species present.

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B. STATIC METHOD FOR ACUTE TOXICITY TESTS WITH PHYTOPLANKTON

1. Introduction

The method described here is designed for analysis of effects of ocean-dumped material on growth of marine unicellular algae. It involves addition of liquid waste or extracts from sludge to algal growth medium, addition of algae to the medium, and measurement of growth for 96 hours.

Because the capability of calculating EC50 values from bioassay data is required by law, dilutions of ocean-dumped material are necessary. As it is impossible to estimate potential algal toxicity (or stimulatory action of each batch of ocean-dumped material, the recommended dilutions may not be sufficient to yield EC50 values in every case). The logistics of algal bioassay are complicated and time consuming. They must be considered carefully before definite requirements are imposed upon testing organizations.

2. Maintenance of Test Organisms

The marine unicellular algal species to be used is Chlorococcum sp. (Milford "C") when only one species is used. If two or three species are to be used in the test, Thalassiosira pseudonana (also known as Cyclotella nana) is the second species of choice, and Porphyridium cruentum the third. These algae may be obtained from the Department of Botany, Culture Collection of Algae, Indiana University, Bloomington, Indiana 47401.

The algae may be ordered as:

Number	Species
819	Chlorococcum (Milford "C")
1269	Cyclotella sp.
637	Porphyridium sp.

The species are to be maintained in stock culture collections in artificial sea water medium. The artificial sea water is prepared by dissolving artificial sea salts (such as Rila Salts, Rila Products, Teaneck, New Jersey 07666) in glass-distilled water to a salinity of 30 parts per thousand (30 grams of salt in 1000 ml of artificial sea water). Add 30.0 ml of metal mix, 2.0 ml of minor salt mix, and 1.0 ml of vitamin mix to each liter. The compositions of the mixes are given in Table 1-B.

Filter (with suction) the sea water medium through a 0.22μ membrane filter (such as that manufactured by the Millipore Corporation, Bedford, Massachusetts 01730, Catalog No. GSWP 047 00). Before filtration, pass 1 liter of 0.1 N HCl and 5 liters of glass-distilled water through the filter. Dispense 200 ml of medium into 500 ml Erlenmeyer flasks and use polyurethane foam plugs to seal the flasks. Autoclave at 120°C and 20 lb pressure for 15 minutes. The flasks must have been cleaned by washing with detergent, soaking in 10% HCl, and rinsing 10 times with distilled water.

Equilibrate at room temperature for one day, and check the pH of medium in a flask especially set up, as above, for this purpose. The pH should be between 7.8 and 8.1. If the pH is not within this range, discard all flasks and make new medium. The pH should fall within this range before a test is started.

Add 10 ml of stock algal culture to each flask and incubate without shaking under 450-500 foot candles illumination at 20° ± 2° C with alternating periods of light (16 hours) and darkness (8 hours). Use standard microbiological techniques for flaming the necks of flasks whenever algae are transferred.

Stock cultures as described above must be renewed every 10 days. They need not be shaken during incubation.

3. Preparation of Test Medium

a. Liquid Waste

If liquid waste is to be tested, it will not be modified before use. When liquid samples are taken for analysis, however, they must be taken in glass containers with Teflon-lined lids. The glassware and liners must be washed with detergent, soaked overnight in 10% HCl, rinsed 10 times with glass-distilled water, rinsed once with acetone, and again rinsed 10 times with glass-distilled water.

Prepare dilutions of liquid waste as follows:

1. Mix 100 ml of liquid waste with 900 ml of artificial sea water that does not contain trace metal, minor salt, or vitamin mixes. This will be considered to be undiluted medium.

2. Add 1 part of 1. to 9 parts of artificial sea water. This is a 10% solution of undiluted medium.

3. Add 1 part of 2. to 9 parts of artificial sea water. This is a 1% solution of undiluted medium.

TABLE 1-B. COMPOSITION OF MIXES TO BE ADDED TO ARTIFICIAL SEA WATER

Mix	Amount
Metal mix:	
$Fe\ Cl_2 \cdot 6\ H_2\ O$*	0.480 g
$Mn\ Cl_2 \cdot 4\ H_2\ O$*	0.144 g
$Zn\ SO_4 \cdot 7\ H_2\ O$*	0.045 g
$Cu\ SO_2 \cdot 5\ H_2\ O$*	0.157 mg
$Co\ Cl_2 \cdot 6\ H_2\ O$*	0.404 mg
H_3BO_3	0.140 g
Na_2EDTA	1.000 g
Distilled water	1 ℓ
Vitamin mix:	
Thiamin hydrochloride	50.0 mg
Biotin†	0.01 mg
B_{12}†	0.10 mg
Distilled water	100 ml
Minor salt mix:	
K_3PO_4	3.0 g
$Na\ NO_3$	50.0 g
$Na_2\ SIO_3 \cdot 9\ H_2\ O$	20.0 g
Distilled water	1 ℓ

*Separate aqueous solutions of these metal salts are maintained at such concentrations that 1 ml of each is added to 1 ℓ of mix.

†Biotin is maintained as 1 mg/100 ml alcoholic stock solution; B_{12} in a 10 mg/100 ml aqueous solution.

4. Add 1 part of 3. to 9 parts of artificial sea water. This is a 0.1% solution of undiluted medium.

b. Sludge

When sludge is to be tested, artificial sea water without trace metals, minor salts, or vitamins will be used as extractant. Salinity of the extractant is 30 parts per thousand and the procedure is:

1. Place a representative portion of the sludge into a 250 ml capacity graduated cylinder, filling to the 250 ml mark. Let the sludge settle overnight (approximately 16 hours). Carefully decant and discard the supernatant.

2. Add 100 ml of the wet settled sludge to a gallon-capacity wide-mouthed jar and add 900 ml of artificial sea water at room temperature. If more growth medium will be required, add more settled sludge and artificial sea water to the jars, but keep the ratio of 100:900 constant. Cap the jars tightly and shake on an automatic shaker at about 100 excursions per minute for 30 min. At the end of the shaking period remove the jar from the shaker, stand it in an upright position and let settle for 1 hour.

3. Filter the supernatant fluid through glass wool, a membrane filter of 5.0μ porosity, and then through a membrane filter of 0.22μ porosity. When the filters clog, replace them as is needed. The filters must be washed before use by passing one liter of 0.1 N HCl and 5 liters of glassdistilled water through them. All glassware associated with filtration must be prepared before use by washing with detergent, soaking overnight in 10% HCl, and rinsing with glass-distilled water.

4. The following solutions will be used in the test:

a. Filtered extract. This will be considered to be undiluted medium.

b. Add 1 part of a. to 9 parts of artificial sea water. This is a 10% solution of undiluted medium.

c. Add 1 part of b. to 9 parts of artificial sea water. This is a 1% solution of undiluted medium.

d. Add 1 part of c. to 9 parts of artificial sea water. This is a 0.1% solution of undiluted medium.

5. After filtration and dilution of liquid or sludge material, add 30.0 ml of metal mix, 2.0 ml of minor salt mix, and 1.0 ml of vitamin mix to each liter and record the pH.

6. Add 48.0 ml of each solution to sterile 125 ml volume Erlenmeyer flasks that were washed with detergent, soaked overnight in 10% HCl, rinsed 10 times with glass-distilled water, rinsed once with acetone, and again

rinsed 10 times with glass-distilled water. Prepare three flasks for each solution and for each algal species used. Use polyurethane foam plugs to seal the flasks.

7. Suggested apparatus for extraction, or their equivalent are:

a. Laboratory shaker, Eberback 6000 with a 605 Utility Box, or equivalent, capable of shaking a 1 gallon container at 100 excursions per minute.

b. Glass jars, wide month, 1 gallon capacity with Teflon lined, screw top lids. Note: It may be necessary to purchase jars and Teflon sheets separately, in which case the Teflon lid liners may be prepared by the laboratory personnel. Jars and lids should be equivalent in quality to those supplied by the Cincinnati Container Corporation, 2833 Spring Grove Avenue, Cincinnati, Ohio 45225. Jars, Cat. No. 120-400-F-0-0-4 (128 oz); Lids, Cat. No. 120-400-White, FTK, PPE. Teflon sheets should be equal in quality to those supplied by the Cadillac Plastic Co., 3818 Red Bank Road, Cincinnati, Ohio 45227.

4. Bioassay

a. Preparation of algae

Four days before the bioassay test is performed, add 5 ml of algal stock culture that is at least 5 days old to 45 ml of sterilized artificial sea water that contains trace metals, minor salts, and vitamins as described under 2. "Maintenance of Test Organisms." Do this in 125 ml capacity Erlenmeyer flasks fitted with polyurethane foam plugs. Incubate these new cultures under 450-500 foot candles from cool white flourescent tubes at 20 ± 2°C. Incubate them on rotary shaker platforms (such as No. G2 shaker fitted with No. AG2-125 platform from New Brunswick Scientific Co., New Brunswick, New Jersey 08903, or equivalent) at 140 ± 10 excursions per minute. The lighting cycle should be 16 hours of light followed by 8 hours of darkness.

On the first day of testing, add 1.0 ml of algal culture to a volumetric flask of 25 ml capacity. Bring to approximately half volume with testing medium, add 2 drops of 10% formalin in growth medium, and bring to full volume with testing medium. Wait 5 minutes.

Shake each flask to attain a homogeneous suspension of cells. Quickly, remove a sample of the homogeneous suspension with a small pipette and fill each side of a Spencer Bright-Line haemocytometer. Be sure that the suspension does not overflow into the troughs of the haemocytometer. At 100X magnification count all cells within and impinging upon the 4 corner 1 mm^2 squares and the central 1 mm^2 of each grid. To find the number of cells in 1 ml the original suspension, multiply the count from the 10 squares by 25,000.

The object of these counts is to determine the dilution required to attain a final concentration of 100,000 cells per ml in the original cell culture. For example, if the number of cells in a ml of culture was 200,000, then the original culture should be diluted 1:1 with test medium to yield 100,000 cells per ml.

b. Growth of algae

Add, using sterile pipettes 2.0 ml of the algal suspension that contains 100,000 cells per ml to the flasks that were prepared with 48.0 ml of test medium.

Place the flasks on rotary shaker platforms and set the platform at 140 ± 10 excursions per minute. Illunination should be from overhead cool flourescent lights. Intensity of light should be between 450 and 500 foot candles with a lighting cycle at 16 hours of light followed by 8 hours of darkness. The temperature should be 20° ± 2°C.

Incubate the shaking cultures for 96 hours. At that time, add two drops of 10% formalin in artificial sea water to each flask, wait five minutes, swirl the cultures to resuspend the cells to a homogeneous suspension and count in a haemocytometer as described above.

c. Untreated controls

Control algal cultures must be grown in untreated medium at the time bioassays on liquid waste or sludge are being done. In this case, untreated medium, with its full complement of metal, vitamin, and minor salt mixes is shaken, filtered and added to flasks in exactly the same manner as when extracting sludge. The cell suspension used to inoculate the untreated growth is prepared exactly as described above except untreated growth medium is used for diluting.

Three flasks are used in growth of controls, and counting is done as described above.

5. Analysis of Results

Calculate the average values for number of algal cells per mililiter in control and each dilution of waste-treated flasks.

An EC50 value is the dilution at which waste material causes 50% reduction in growth. In order to estimate this value, inspect the average values to learn if numbers of algal cells in the waste-treated flasks were (1) less than half that in the untreated control flasks, and (2) more than half that in the untreated control flasks. If not, then an EC50 value cannot be determined.

If average cell counts among the dilutions fall greater and less than half of those of the controls, then EC50 values must be estimated. Using semilogarithmic coordinate paper, plot the average cell count for a dilution that yielded more than half, and the average cell count in a dilution that yielded less than half of the average cell count of control flasks. The dilution should be plotted on the logarithmic axis and the percentage of growth in relation to the control on the arithmetic axis. Draw a straight line between the two points. The concentration at which this line crosses the 50% growth line is the EC50 value.

C. STATIC METHOD FOR ACUTE TOXICITY TESTS WITH BRINE SHRIMP, Artemia Salina

1. Introduction

The test organism is the brine shrimp, Artemia salina. Artemia are ecologically insignificant and may be more resistant to many toxicants than are natural zooplankton. However, the lack of culture and confinement procedures for natural zooplankton species limit their use even during periods of peak abundance.

Twenty-four hour nauplii larvae are exposed to 6 concentrations of the test material for 48 hours. Each concentration and control is replicated; one is aerated and one is unaerated. The concentration effective against 50% of the animals (EC50) can then be determined by inspection of a graph of the data. The toxicity of waste to brine shrimp should be compared to that with appropriate sensitive marine organisms which include, when possible, indigenous species at the dumpsite. Brine shrimp should not be used alone.

2. Selection and Preparation of Test Organisms

Artemia was selected as the test organism due to its universal and nonseasonal availability and relative low cost of the assay. The eggs of Artemia can be purchased from local pet stores and stored in a dessicated but viable state for long periods of time, and the required number of organisms can readily be obtained at any time through the use of proper hatching procedures. Eggs from the San Francisco Bay area are preferable.

A rectangular tray (glass or enamel) having approximately 1300 square centimeters (cm) of bottom surface is suitable for hatching Artemia eggs. Divide this tray into two parts by a partition that extends from the top down to about 2.0 to 1.5 cm from the bottom. This partition may be of any opaque, biologically inert material (a pasteboard strip, sealed with paraffin wrapping is satisfactory). Raise one end of the tray about 1.5 cm and add 3 liters of sea water formulation. Spread approximately 0.5 g of brine shrimp eggs in the shallow end of the tray. Cover this end of the tray with a piece of cardboard to keep the eggs in darkness until hatching is complete. After 20-23 hours incubation, direct a narrow beam of light across the uncovered portion of the tray. Brine shrimp are phototactic and will swim beneath the partition into the illuminated end of the chamber. The Artemia concentrated in the beam of light can be easily collected with the use of collecting pipette or siphon connected to a 30.5 cm (12 inches) rubber tube and mouthpiece and transferred to a beaker or shallow dish containing a small amount of salt water.

3. Salt Water

For acute toxicity tests with Artemia, a practical criterion for an acceptable salt water is that healthy test animals will survive in it for the duration of hatching and testing without showing signs of stress. Salt water should be prepared from commercially available formulations or from ingredients listed on page 5, using deionized or glass-distilled water. Deionized or distilled water should be used to dilute the salt water to a salinity of 30 parts per thousand ($^o/oo$).

4. Recommended Procedure for Testing Material

a. Experimental Design

The recommended test procedure consists of two separate 48-hour bioassays with a control and six concentrations of the material to be tested. One 48-hour bioassay will be without aeration and the second will be with aeration. In the latter, containers will be aerated with clean air at the rate of 100 ± 15 bubbles per minute delivered from a glass tube 1 millimeter (mm) inside diameter.

There must be at least 20 control animals and at least 20 animals must be exposed to each concentration or dilution of the material to be tested, but they may be divided between two or more test containers. The use of more animals and replication of treatments is desirable. If replicates are used, they should be true replicates with no water connection between the replicate test containers. Stratified randomization of the treatments (random assingment of one test container for each treatment in a row followed by random assignment of a second test container for each treatment in another or an extension of the same row) or total randomization of the treatments is recommended. A representative sample of the test animals should be impartially distributed to the test containers, either by adding one (if there are to be less than 11 animals per container) or two (if there are to be more than 11 animals per container) test animal to each container, and then adding one or two more to each test container, and repeating the process until each test container has the desired number of test animals in it. Alternatively, the animals can be assigned either by total randomization or by stratified randomization (random assignment of one animal to each test container, random assignment of a second animal to each test container, etc.).

Every test requires a control which consists of the same salt water, conditions and animals as are used in containers with test material. A test is not acceptable if more than 10% of the control animals die.

b. Temperature

Test water temperature must be maintained within 1°C of the water temperature listed on page 65.

c. Salinity

Test water salinity should be 30 ^{o}/oo before the material to be tested is added.

d. Test Containers

Use Carolina culture dishes (or their equivalent) having dimensions approximately 9.0 by 6.5 centimeters.

Test containers must be cleaned before use. New containers must be washed with detergent and rinsed with 10% hydrochloric acid, acetone, and tap or other clean water. At the end of every test, if the test containers are to be used again, they should be (1) emptied, (2) rinsed with water, (3) cleaned by a procedure appropriate for removing the toxicant, e.g., acid to remove metals and bases; detergent, organic solvent, or activated charcoal to remove organic compounds; and (4) rinsed with water. Acid is useful for removing scale and hypochlorite (bleach) is useful for removing organic matter and for disinfecting. All test containers must be rinsed with salt water just before use.

e. Preparation of Material to be Tested (See other section of manual on this subject.)

f. Concentrations

Dilutions of samples, by volume, of 10% (100,000 ppm, 100 ml/ℓ), 1% (10,000 ppm, 10 ml/ℓ), 0.1% (1,000 ppm, 1 ml/ℓ), 0.01% (100 ppm, 0.1 ml/ℓ), 0.001% (10 ppm, .01 ml/ℓ), and 0.0001% (1 ppm, 0.001 ml/ℓ) are recommended as initial test concentrations.

The highest concentration (dilution) will be prepared as follows: 9 volumes of salt water will be added to 1 volume of the stirred sample. (Adequate space should be reserved in the test container for stirring and addition of animals.)

Each succeeding concentration will be prepared by a similar 1-in 10 serial dilution from the previous test container. Adequate stirring of the contents of the test container is essential before each dilution.

g. Aeration of One Bioassay

Following diolution, aeration of one set of concentrations should begin using 100 ± 15 bubbles per minute delivered through a 1 mm ID glass tube.

h. Transfer of Animals

Animals must be added to the test containers within 1 hour after the proper dilutions of the material to be tested have been made and aeration of one set of test containers begun.

i. Feeding

The organisms must not be fed while in the test containers.

j. Measurements

The dissolved oxygen concentration, pH, and temperature must be measured (1) before adding animals and (2) at 24-hour intervals thereafter in the highest and lowest concentration and the control of both aerated and unaerated bioassays. Additional measurements are required in containers in which animals die. Water samples should be taken midway between the top, bottom, and sides of the test containers and should not include any surface scum of material stirred up from the bottom or sides.

k. Observations

At a minimum, the number of dead or affected animals must be recorded at 24-hour intervals throughout the test. More observations are often desirable, especially near the beginning of the test. Dead animals must be removed as soon as they are observed.

To count the dead animals accurately and with relative ease, place the test dishes on a black surface and hold a narrow beam of light parallel to the bottom of the dish. By searching the surface, bottom, and intermediate areas, account for the live animals. The use of a dropping pipette or medicine dropper to remove the live animals facilitates the counting and decreases the confusion of trying to count moving larvae. Due to the turbidity that may accompany the higher concentrations of sludge materials, a 5 minute settling period may be helpful. Pouring the contents of the test container into a clean container with a larger surface area may make the animals more visible. Various magnifying devices may also be of use.

The adverse effect most often used to study acute toxicity with aquatic animals is death. However, death may not be easily determined for some Artemia, and so an EC50 (effective concentration to 50% of test animals) is often measured rather than an LC50 (lethal concentration to 50% of test animals.) The effect generally used for determining an EC50 is immobilization, which is defined as the inability to move except for minor activity of appendages, or loss of equilibrium.

5. Calculations and Reporting

At the end of the test period, the bioassays are terminated and the LC50 or EC50 values are determined.

a. Calculations

An LC50 is a concentration at which 50% of the experimental animals died and an EC50 is a concentration at which 50% of the experimental animals were affected. Either may be an interpolated value based on percentages of animals dying or affected at two or more concentrations. Estimating the LC50 or EC50 by interpolation involves plotting the data on semilogarithmic coordinate paper with concentrations on the logarithmic axis and percentage of dead or affected animals on the arithmetic axis. A straight line is drawn between two points representing death or effect in concentrations that were lethal to or effective against more than half and to less than half of the organisms. The concentration at which the line crosses the 50% mortality or effect line is the LC50 or EC50 value. If 50% of the test animals are not affected by the highest concentration, the percent affected should be reported.

b. Reports

Any deviation from this method must be noted in all reports of results. A report of the results of both aerated and unaerated tests must include:

1. name of method, author, laboratory, and date tests were conducted;

2. a detailed description of the material tested, including its source date and time of collection, composition, known physical and chemical properties, and variability of the material tested;

3. the source of the salt water, date prepared and method of preparation;

4. detailed information about the test animals, including name and source;

5. a description of the experimental design, the test containers, the volume of test solution, the way the test was begun, the number of organisms per concentration, and the loading;

6. definitions of the criteria used to determine the effect and a summary of general observations on other effects or symptoms;

7. percent of control organisms that died or were affected in each test container during the test;

8. the 24- and 48-hour LC50's or EC50's;

9. methods used for and the results of all dissolved oxygen, pH, and temperature measurements; and

10. any other relevant information.

D. METHODS FOR THE CULTURE AND SHORT TERM BIOASSAY OF THE CALANOID COPEPOD *Acartia tonsa*

1. Introduction

The methodology described in this section is designed to provide bioassay data on the effects of a toxicant on a marine copepod. The techniques described have been used for several years by EPA and represent the synthesis of many researchers' efforts both from government and universities.

The bioassay format recommended is not new and has been employed in aquatic toxicology for many years. Basically, dose response curves are constructed from mortality rate data collected from 24, 48, 72, and 96-hour exposure observations. While these observation intervals should be considered a requirement, more frequent observations or exposures longer than 96 hours should be included when the research design dictates. From the above observations, estimates of LC10, LC50, LC100 and confidence limits can be determined (Litchfield and Wilcoxon, 1949; Finney, 1964, 1971; Standard Methods, 1971, 13th Edition).

2. Collection and Preparation of Sea Water

There are two distinct aspects to the sea water requirements for this bioassay. The sea water for these functions should, if possible, be collected from the study area. First, the sea water when adjusted to 30 °/oo salinity and 20° C must support survival of the adult copepod *Acartia tonsa* for the 96-hour bioassay period.

A second and more demanding requirement of the sea water is that, with the proper enrichments, it supports growth of the food algae and the complete life cycle of the test species. If no visible suitable natural sea water is available that satisfies these requirements a synthetic sea water formulation may be employed, (Table 1-D).

Niskin or Van Dorn samplers can be used to collect sea water from 3 to 10 meters depth to avoid surface contamination. Collected sea water can be transported to the laboratory in glass or polyethylene carboys that have been aged in sea water. Upon return to the laboratory the water is filtered through a 1.0μ acid washed filter (glass fiber, cellulose, acetate, nylon or polycarbonate) to remove particulate matter and the water stored at 4° C in the above containers.

Measurements of salinity, dissolved oxygen and pH should be recorded at the time of collection.

TABLE 1-D. SYNTHETIC SEA WATER FORMULATION*

Chemical	g/ℓ
NaCl	24.00
Na_2SO_4	4.00
$CaCl_2.2H_2O$	1.47
$MgCl_2.6H_2O$	10.78
KCl	0.70
H_3BO_3	0.03
$NaHCO_3$	0.20

---

*Medium is modified from Kester et al. (1967). Medium has salinity of 34 ^{o}/oo and pH 8.0 and must be adjusted to 30 ^{o}/oo with distilled or deionized water. Trace metal contaminants from major salts must be eliminated by ion-exchange stripping (Davey et al. 1970). Na_2EDJA (300 mgs/ℓ) may be used for holding and culture but must be omitted in bioassay studies with trace metals.

3. Algal Food Cultures

Although a variety of algal diets have been used for copepod cultures (Zillioux & Wilson 1966; Heinle 1969; Katona 1970; Nassogne 1970) the following modification of Wilson & Parrish (1970) has been used in our laboratory quite successfully (Table 3-D). We have added *Skeletonema costatum* because it is a naturally occurring food for *Acartia tonsa*.

TABLE 2-D. SEA WATER AND STERILITY ENRICHMENTS

(A) Sea water enrichments for stock algal culture maintenance (After Guillard and Ryther, 1962):

Item	Amount
$NaNO_3$	75 mg/liter
$NaH_2PO_4.H_2O$	5 mg/ℓ
$Na_2SiO_3.9H_2O$	10 mg/ℓ
Vitamins:	
Thiamine HCl	0.10 mg/ℓ
Biotin	0.50 μg/ℓ
B_{12}	0.50 μg/ℓ
Trace Metals:	
$CuSo_4.5H_2O$	0.002 mg/ℓ
$ZnSO_4.5H_2O$	0.004 mg/ℓ
$CoCl_2.6H_2O$	0.002 mg/ℓ
$MnCl_2.4H_2O$	0.036 mg/ℓ
$NaMoO_4.2H_2O$	0.001 mg/ℓ
Fe-sequestrine	1.0 mg (0.13 mg Fe)/ℓ
Buffer:	
TRIS-500 mg/ℓ @ pH 7.8-8.2	

(B) Sterility enrichments to be added to enriched sea water medium above before autoclaving:

Sodium Glutamate	250 mg/ℓ
Sodium Acetare	250 mg/ℓ
Cycline	250 mg/ℓ
Nutrient Agar	50 mg/ℓ
Sucrose	250 mg/ℓ
Sodium	250 mg/ℓ
L & D Alanine	250 mg/ℓ

TABLE 3-D. COMPOSITION OF ALGAL DIET AND RECOMMENDED CONCENTRATION FOR FEEDING, EGG LAYING, AND NAUPLIAR FEEDING

Item	Adult & Copepodite	Naupliar	Egg Laying
Skeletonema costatum	5.0 X 10^6	5.0 X 10^5	1.5 X 10^7
Thalassiosira psuedonana	7.0 X 10^6	7.0 X 10^5	2.1 X 10^7
Isochrysis galbana	5.0 X 10^6	5.0 X 10^5	1.5 X 10^7
Rhodomonas baltica	3.0 X 10^6	3.0 X 10^5	9.0 X 10^6
Total cells/liter	2.0 X 10^7	2.0 X 10^6	6.0 X 10^7

These algae are grown axenically in filtered natural or synthetic sea water at 30 ^{o}/oo salinity and 20° C with 2500-5000 lux continuous illumination or 14L:10D. The nutrient enrichments are modifications of those of Guillard and Ryther (1962), (Table 2-D).

Algal cultures may be grown either in standard test tube or flash cultures if desired; or in the fill and draw semi-continuous system described below.

Enriched sea water is dispensed into either screw-capped test tubes (50 ml) or erlenmeyer flasks fitted with Teflon lined caps. After standard autoclaving (15 min. @ 15 psi & 250°F) the medium is allowed to cool and equilibrate with atmospheric gases for 48 hours. Sterility checks are made on each set of autoclaved medium by randomly selecting a representative number of tubes or flasks and inoculating 1 ml of their contents into tubes of sterility check medium (Table 2-D). Caps are tightened and the inoculated tubes stored in the dark for up to 2 weeks. The appearance of turbulence or opalescence in the test medium indicates the presence of contamination.

Tubes or flasks are inoculated with each alga on a regular basis to continually provide a log-phase, high density food source, the frequency of which will be determined from interpretation of algal growth curves. The cultures should be harvested at their maximum log-growth phase cell density. While the above system does work, it requires many manipulations which can result in culture contamination. It is very time consuming since it requires frequent cell counts and a large turnover of glassware.

The recommended algal culture system is of a fill-draw type where cultures are easily maintained near their maximum log-phase cell density and growth rate, (Figure 1-D). It is then a simple matter to draw off and

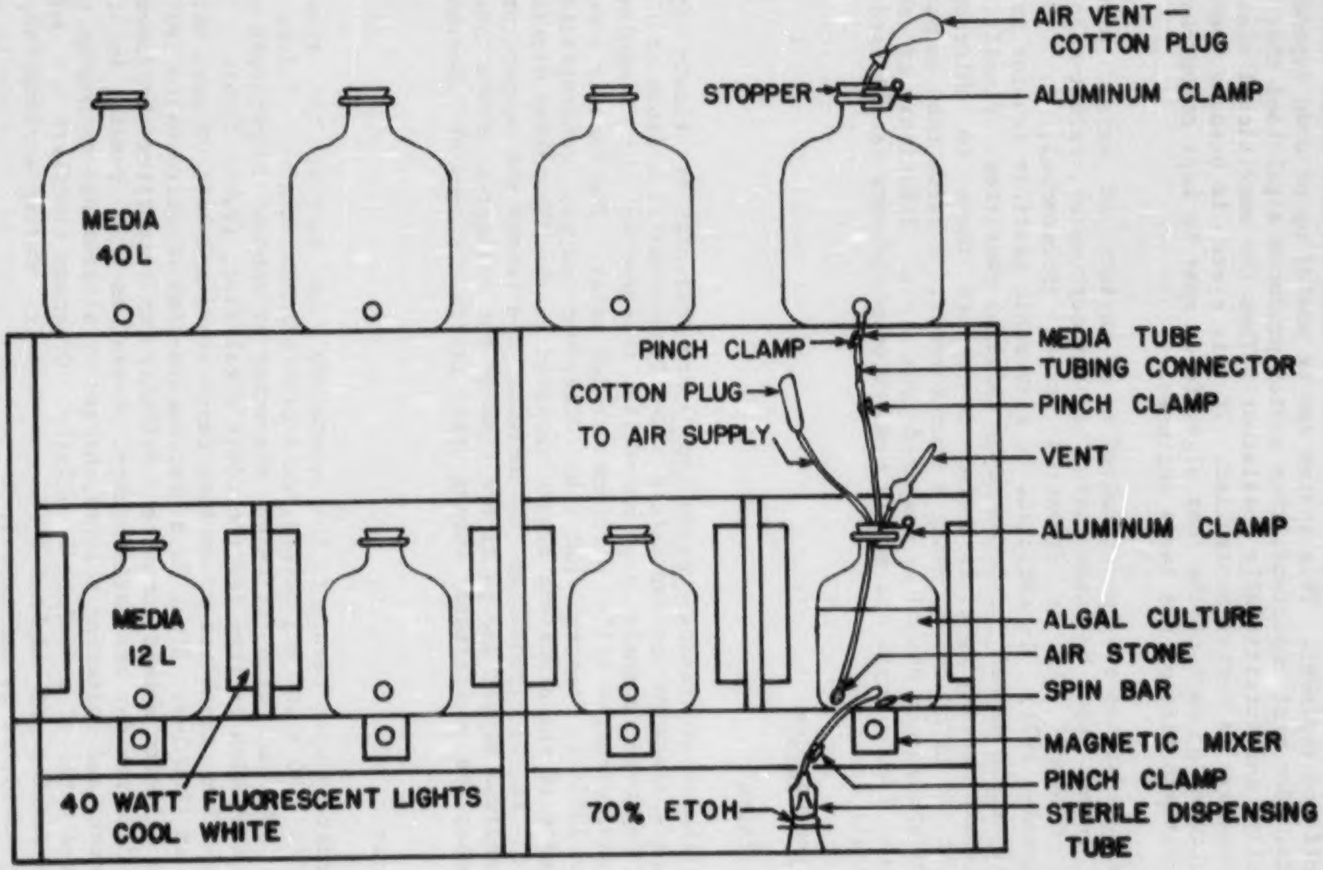

AIR VENT — COTTON PLUG
STOPPER
ALUMINUM CLAMP
MEDIA 40L
MEDIA TUBE
PINCH CLAMP
TUBING CONNECTOR
COTTON PLUG
PINCH CLAMP
TO AIR SUPPLY
VENT
ALUMINUM CLAMP
MEDIA 12 L
ALGAL CULTURE
AIR STONE
SPIN BAR
MAGNETIC MIXER
PINCH CLAMP
40 WATT FLUORESCENT LIGHTS COOL WHITE
70% ETOH
STERILE DISPENSING TUBE

Figure 1-D. Algal culture.

replace a constant volume with fresh medium so that within 24 hours the culture will have reached the same cell density. When longer than 24 hour intervals occur between harvests, proportionally greater amounts of culture are drawn off and replaced. This system can be scaled up or down depending on food needs. But most important, this system produces algal food that is physiologically and nutritionally consistent. Thus the nutritional history of the test species is better controlled. If this system is used, a series of tube cultures of each of the four algal foods must be kept concurrently in case of contamination of the large cultures.

Algal cell densities may be determined in a variety of ways. Direct microscopic counts can be made using a haemocytometer, Palmer-Maloney chamber, or Utermohl chamber (inverted scope) (Schwoerbel, J. 1970) (Standard Methods, 1971). In addition, an electronic particle counter is an accurate and rapid method for determining unialgal densities. Finally, if manual counts are necessary, it is useful to relate these to chlorophyl absorbance at 440 mμ or 665 mμ using a spectrometer. A curve that compares cells/ml with absorbancy should be prepared from serial dilutions of each algal culture. Then a rapid and simple measure of absorbancy can be used to replace the cell count.

4. Zooplankton Culture

a. Collection

Zooplankton (including Acartia tonsa) are collected by slowly (≤ 4 km/hr) towing a plankton net (aperature 150-250 microns) at a depth of 1 to 3 meters. Captured animals are carefully transferred to insulated containers three-fourths filled with ambient sea water. The density should not exceed ca.250/ℓ to assure that the dissolved oxygen concentrations remains adequate if the organisms aren't returned to the laboratory within 1 to 2 hours. It is imperative to measure and record the temperature, salinity, dissolved oxygen and pH at the time of the collection since these parameters must be maintained during the initial stages of laboratory culture.

b. Holding

Immediately upon return to the laboratory the samples are transferred to 2.3/ℓ (190 x 100 mm) borosilicate crystallizing dishes. Volume is adjusted to 2000 ml with filtered sea water at ambient temperature and salinity and each dish is then fed the adult algal diet, (Table 3-D). The cultures are then incubated at ambient temperature and 14L:10D cool white illumination at 1000 lux. After 24 hours, acclimation of cultures to 20° C and 30 ^{o}/oo salinity should commence. Salinity and temperature increments of 5°C and 5 ^{o}/oo per day are satisfactory. Organisms can remain in the original vessel and culturing volumes changed by alternately siphoning and then adding sea water of a different salinity. Organism transfers are made by either pipetting or siphoning to new vessels. During acclimation, a daily feeding scheduled is maintained.

Holding and acclimating can also be accomplished by adding the tow collections to large aspirator bottles equipped with low rpm stirring motors. Organism density should be adjusted to 1:10 ml of culture volume.

c. Sorting and Identification

The Plankton tow contains a mixture of species from which *Acartia tonsa* must be isolated. For basic information on the taxonomy and biology of the genus *Acartia* and other coastal calanoids the following papers are recommended (Conover, 1956; Heinle, 1966, 1969; Wilson, 1932; Rose, M., 1933; Fraser, J.H. & Hansen, V. Kr., Ed., *Series Fiches D'Identification Du Zooplancton*).

To facilitate capture, the culture volume is reduced from 2000 ml to 500 ml by slowly siphoning sea water using 150 micron plankton netting over the siphon intake. Individual adult organisms are carefully drawn up into a wide bore (≥ 2 mm) transfer pipette and individual animals placed in depression slides, identified, and transferred to food enriched filtered sea water at 30 $^{o}/oo$ and 20°C. Care should be taken to exclude all nauplii and juvenile forms in order to eliminate contaminant species.

d. Mass Culture

The objective of this system is to provide large quantities of *Acartia tonsa* of standard age for short-term bioassays.

The mass culture unit is derived from culture systems used by Mullin and Brooks (1967) and Frost (1972). The culture vessel is a pyrex aspirator bottle whose size can range from 3.5 to 12.0 gallons depending on the number of copepods needed. The contents are gently mixed by a low rpm motor (≤25 rpm) mounted above the culture vessel. The slow mixing maintains algal food in suspension where these planktonic copepods normally feed. It must be emphasized that water movement is gentle and free of vorticies such as produced by magnetic stirrers (Figure 2-D). Cool white flourescent lights provide 2000 lux illumination incident to the culture surface on a 14L:10D cycle.

Acartia tonsa females are capable of producing in excess of 30 eggs per female per day when fed the food ration recommended in Table 3-D (Wilson & Parrish, 1971). Thus if 250 or more gravid females are brooded, theoretically over five thousand eggs will be produced within 24 hours. For this potential number of adults, a 40/ℓ culture vessel would be desirable. Generally, the relationship between culture volume (mls) and organism density is 10ml:1.

Fifty to one hundred gravid females are placed in each of three to five generation cages (Figure 3-D) immersed in 2.3/ℓ crystallizing dishes containing ca.2000 ml of sea water and fed at 3 times the usual concentration (Table 3-D). The generation cage allows the eggs to pass through the net and hatch eliminating the possibility of cannibalism by adults. After 24 hours the adults are removed by gently lifting each

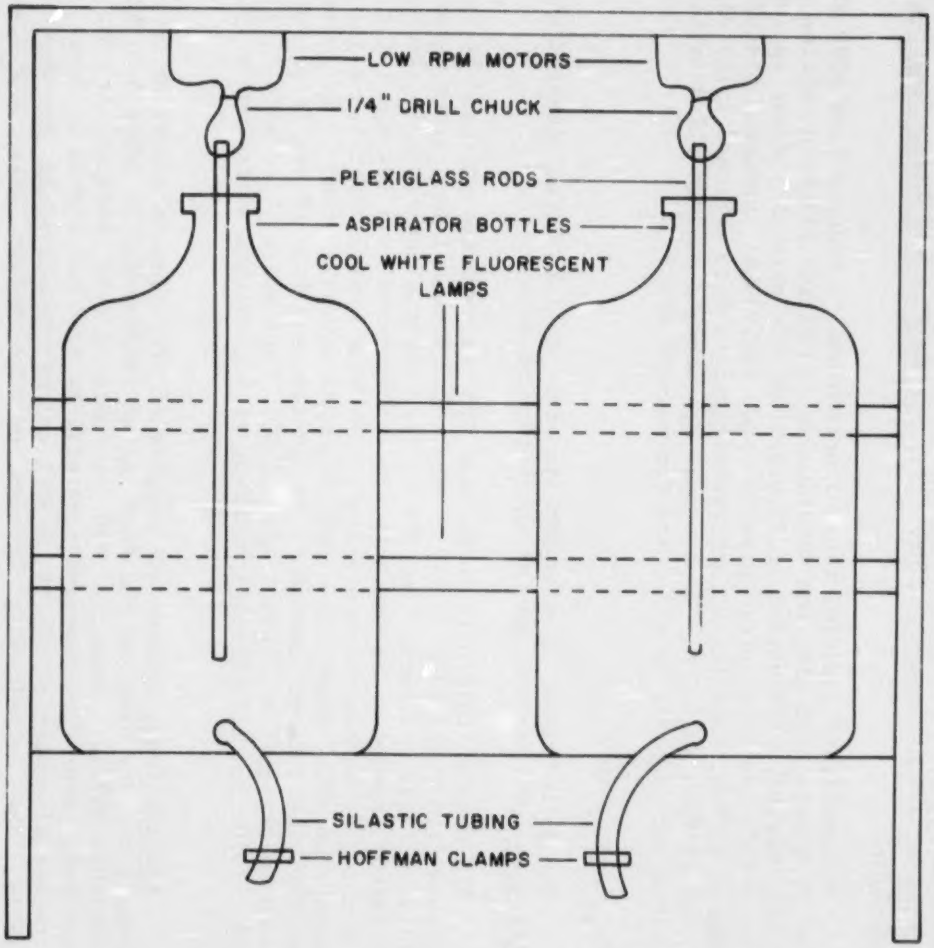

LOW RPM MOTORS
1/4" DRILL CHUCK
PLEXIGLASS RODS
ASPIRATOR BOTTLES
COOL WHITE FLUORESCENT LAMPS
SILASTIC TUBING
HOFFMAN CLAMPS

Figure 2-D. Mass copepod culture (static).

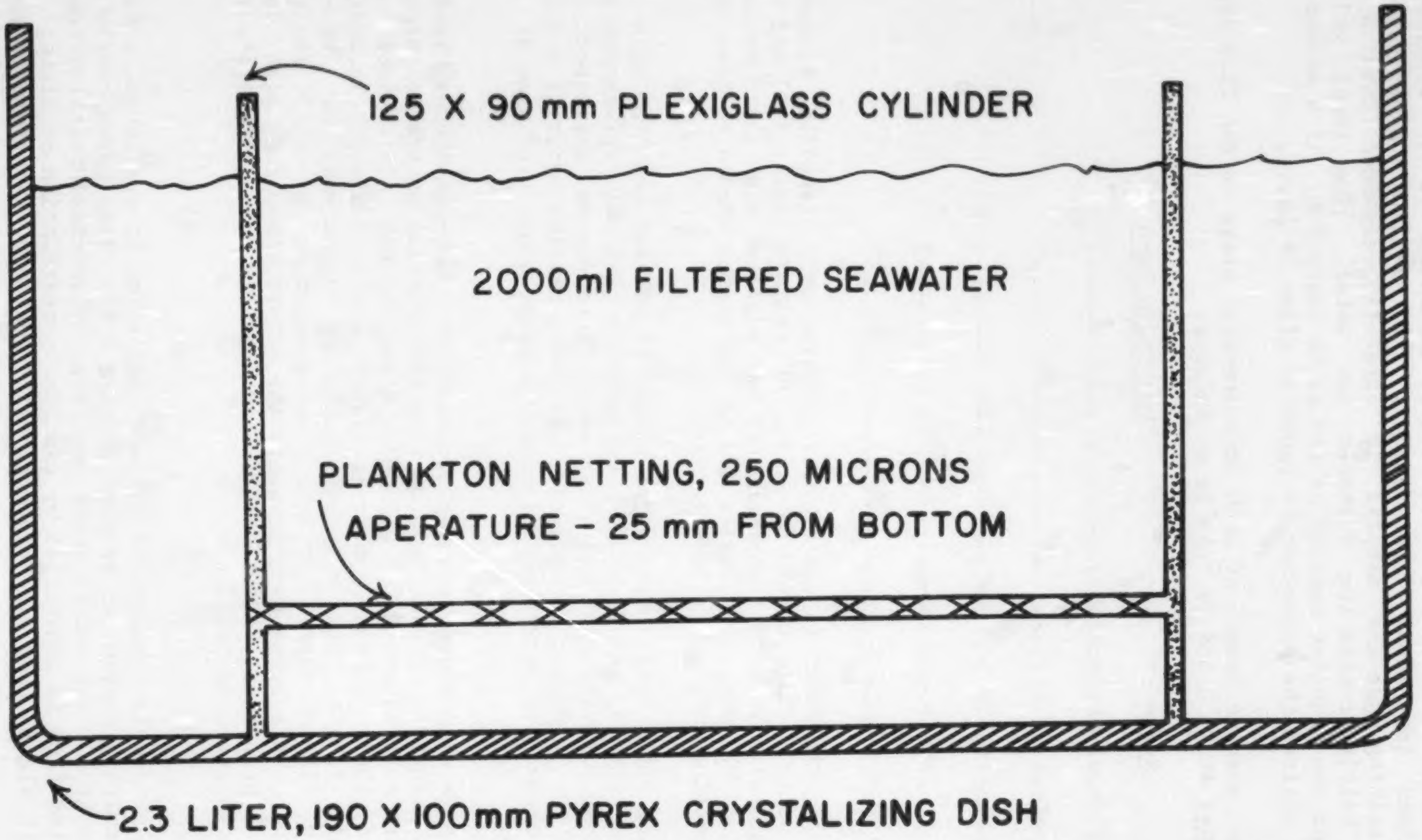

125 X 90 mm PLEXIGLASS CYLINDER
2000 ml FILTERED SEAWATER
PLANKTON NETTING, 250 MICRONS APERATURE - 25 mm FROM BOTTOM
2.3 LITER, 190 X 100 mm PYREX CRYSTALIZING DISH

Figure 3-D. Generation cage (after Heinle).

generation cage out of the dish and quickly immersing it in another dish with 3 times the usual food density. The remaining sea water from all dishes containing eggs and nauplii is carefully siphoned into a glass aspirator bottle containing filtered sea water. The final volume is adjusted and the naupliar culture is fed as in Table 3-D. If a second mass culture is desired the procedure is repeated after 24 hours.

The average length of each developmental stage in the life cycle of Acartia tonsa at 20°C and 20 ^{o}/oo is as follows:

Stage	Length in Days
Egg (newly oviposited)	1
Nauplius (6 instars)	7
Copepodite (6 instars)	6
Adult (until gravid)	3
Total life cycle	17

During the first 6 days of mass culture only naupliar stages are present. Daily feeding should be at 2×10^6 cells/ℓ (Table 3-D) and 50% of the culture medium should be siphoned off and replaced with clean medium on the 3rd and 7th days. The intake end of the siphon should be covered with 60 micron netting to prevent loss of nauplii.

After the 7th day copepodites should be present and from this point on, feeding should be 2×10^7 cells/ℓ/day with 50% replacement of the culture volume with filtered sea water every third day as described above. Within 16 to 17 days the population will have reached maturity and can be bioassayed or used to start new cultures. Average adult life span at 20° C is ≤30 days.

We have also found it useful to maintain a non-age-standardized mass culture in reserve. Gravid females from the original generation cages are used to start a 12 liter (3 1/2 gallon) system, fed the adult food ration and 50% of their culture water replaced every 3rd day. In addition, approximately 1/3 of the culture (including organisms) is harvested periodically (10-14 days) to keep the population at ca. 50 adults and copepodites/ℓ. This precaution is worth the effort since the high density cultures have occasionally "crashed" for no apparent reason. A protocol for this system is given in Table 4-D.

If a constant source of filtered sea water is available, a flowing water mass culture system can be used (Figure 4-D). This sytem consists of a constant head tank which feeds two large cylindrical reaction vessels. Dilution water flow is controlled by capillarly restriction or clamps. The four specie algal food is proportionally metered by peristatic pump to

TABLE 4-D. PROTOCOL FOR MASS COPEPOD CULTURE

Step	Day of culture	Age-standardized	Non-age-standardized
1	1-3	Naupliar diet daily (Table 3)	Adult diet daily (Table 3)
2	4	Replace 50% culture medium with filtered s.w. retaining organisms. Feed as in 1	As in step 2. Feed as adults.
3	5-6	As in 1	As in 1
4	7	Repeat step 2	Repeat step 2
5	8-9	As in 1	As in 1
6	10	Repeat step 2 Adult diet	Repeat step 2
7	11-12	Adult diet daily	As in 1
8	13	Repeat step 6	Repeat step 2
9	14-15	As in 7	As in 1
10	16	Repeat step 6	Harvest 33% of culture including organisms. Transfer remaining 67% to a clean carboy by siphon* & add filtered s.w. to volume.
11	17-18	As in 7	Repeat steps 1-10
12	19	Harvest for Bioassays	---

*Rate of siphoning is controlled by difference in "head pressure". Do not constrict the siphon tube or animals will be damaged.

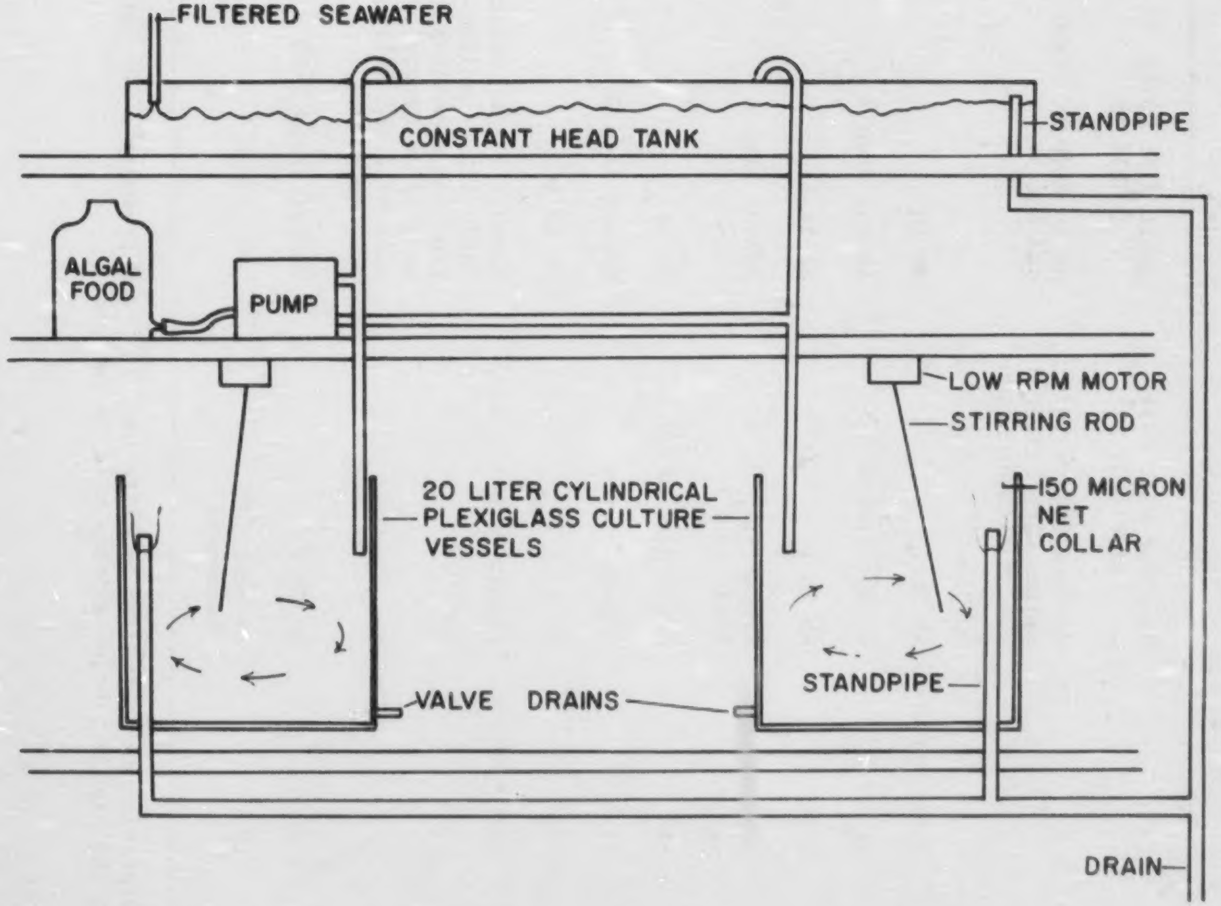

FILTERED SEAWATER
CONSTANT HEAD TANK
STANDPIPE
ALGAL FOOD
PUMP
LOW RPM MOTOR
STIRRING ROD
20 LITER CYLINDRICAL PLEXIGLASS CULTURE VESSELS
150 MICRON NET COLLAR
VALVE
DRAINS
STANDPIPE
DRAIN

Figure 4-D. Mass copepod culture (flowing).

provide a constant cell density of $2-5 \times 10^7$ cells/ℓ. This cell density can sustain culture densities in excess of 100 adults/copepodites/ℓ though harvesting is recommended to keep the density at 50/ℓ.

The reaction vessels are 30 cm high with 30 cm diameter and a 25 cm standpipe. This provides approximately 18ℓ culture volume. The standpipe has a collar of 150 micron nitex net which effectively retains both eggs and nauplii even though they are considerably smaller. Too fine a net produced excessive clogging. It is likely that bacterial and algal growths reduce the effective mesh size to occlude particles as small as 50 microns. This net collar requires brushing periodically to maintain effective drainage. The reaction vessels are illuminated as in the static system and are equipped with low rpm (≤25) motors to maintain the population in suspension. The dilution rate is approximately 10 ml/min which results in a effective replacement of 50% of the culture volume every 24 hours although the total volume pumped is 80% of the reaction volume. Flow rates >10 ml/min can be used, but with caution, as one doesn't want to wash out eggs and/or nauplii.

This basic culture design has been scaled down to 4-liter reaction vessels and is presently being used for long-term bioassay studies on population structure and reproduction.

e. Harvesting

Mass cultures of copepods that have reached the adult stage are harvested for use in bioassays as follows: the culture volume is reduced by 75% using a slow siphon whose intake is covered with 60 micron plankton netting. The remaining 25% of the culture including organisms is carefully transferred to 2.3ℓ pyrex crystallizing dishes (ca. 2000 ml/dish). This transfer is critical and is best performed as follows. Because of the fragility of the organism, it is not advisable to constrict the discharge tube to reduce flow. The discharge flow through the ventral tubulation on the aspirator is controlled by minimizing the head pressure between the culture vessel and the crystallizing dish. A slow flow minimizes turbulence and the opportunity for organisms to collide with vessel walls.

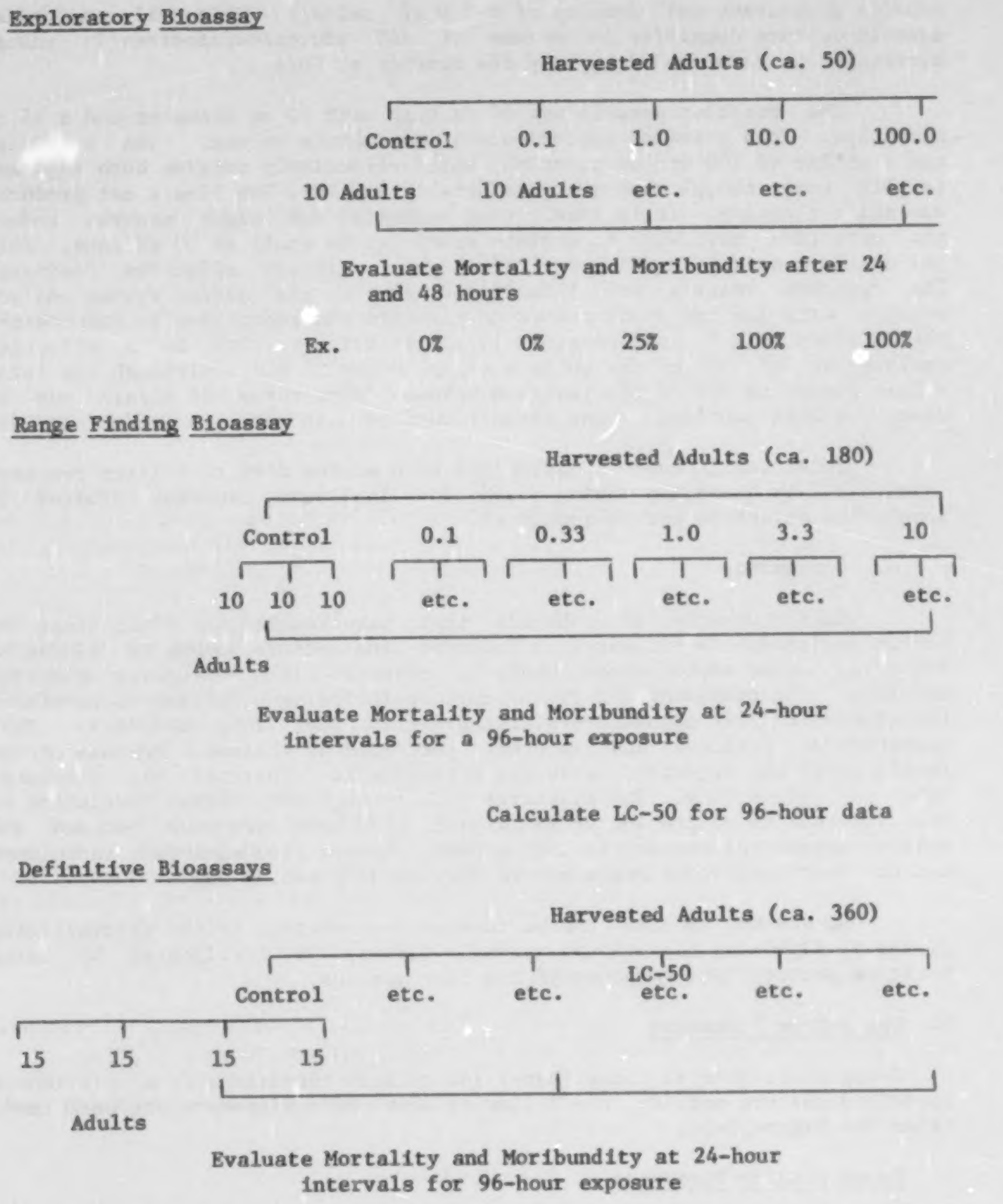
Harvested animals can be further concentrated in the crystallizing dishes by siphoning the culture medium. Capture is facilitated by using positive phototactive response of the test species.

5. Short-Term Bioassays

Using adult *Acartia tonsa* (Dana) and culture conditions from previously described culture methods, the following short-term bioassays are performed. (Also See Figure 5-D.)

Range Finding Bioassays

1) Ten adult *Acartia* are tested per replicate with 3 replicates required per test concentration and control. Feeding is omitted for the duration of the assay. A solvent control must be included when appropriate.


Exploratory Bioassay

Harvested Adults (ca. 50)
Control
0.1
1.0
10.0
100.0
10 Adults
10 Adults
etc.
etc.
etc.

Evaluate Mortality and Moribundity after 24 and 48 hours

Ex.
0%
0%
25%
100%
100%

Range Finding Bioassay

Harvested Adults (ca. 180)
Control
0.1
0.33
1.0
3.3
10
10
10
10
etc.
etc.
etc.
etc.
etc.
Adults

Evaluate Mortality and Moribundity at 24-hour intervals for a 96-hour exposure

Calculate LC-50 for 96-hour data

Definitive Bioassays

Harvested Adults (ca. 360)
Control
etc.
etc.
LC-50
etc.
etc.
etc.
15
15
15
15
Adults

Evaluate Mortality and Moribundity at 24-hour intervals for 96-hour exposure

Figure 5-D. Bioassay protocol.

2) Test container will consist of a suitable flat bottom borosilicate glass dish containing 100 ml sea water. The depth of medium must be ≥ 2.0 cm.

3) Toxicant Concentration Selection

Generally a broad range of concentrations covering at least four orders of magnitude is chosen initially. This is followed by a progressive bisection of intervals on a logarithmic scale (see Standard Methods, 1971).

4) Toxicant Administration

a. Water miscible toxicants immediately prior to the addition of the test species.

b. Water immiscible toxicants will be dissolved in a suitable solvent prior to addition to the test medium. Solvent evaluation must be performed to insure solvent concentrations used are not toxic.

5) Ten adult Acartia are captured from stock cultures with a wide bore transfer pipette and transferred to a 20 ml beaker containing undosed filtered sea water (ca. 5 ml). Adjust the final volume of this beaker to 15 ml. The animals and the 15 ml of medium are added to 85 ml of toxicant-dosed medium by immersing the beaker and gently rinsing.

6) Exposure period will be 96 hours. The number of dead and moribund, copepods will be observed and recorded at 24, 48, 72, and 96 hours of exposure. To ascertain if a motionless animal is dead it is gently touched with a sealed glass capillary probe. Dead animals are removed at each observation point. Control mortalities in excess of 15% invalidate the experiment.

7) At each observation period measures of dissolved oxygen and pH should be made particularly if wastes contain high amounts of organic matter. Since the test species is very sensitive to agitation, these measurements must be made on a series of concurrently prepared uninoculated samples at all test concentrations.

Definitive Short-Term Bioassay

1) General culture conditions and handling will follow previous discussion. The specifications for this assay are as follows.

2) Fifteen adults are to be tested per each of 4 replicates per toxicant concentration and control.

3) Test vessels will be as described above.

4) Concentration ranges for toxicant will be chosen so as to include at least two levels above and below the 96-hour LC50 determined from range finding bioassays.

5) Exposure and data collection will be as above.

6) Calculations, data presentation are as described in Standard Methods (14th Ed.) pp. 565-577. Alternate methods of data presentation are desirable, particularly the application of confidence limits. See Litchfield and Wilcoxon, 1949; and Finney, 1964, 1971.

The bioassay methodology is at best a general framework which is subject to modifications as determined by the type of toxicant and the experimental design.

For example, in assays with toxicants that readily adsorb to container walls and fail to remain in solution, transfer of organisms to freshly dosed media is required. The frequency of transfer being determined after rates of solubility and adsorption have been determined.

6. Standard Toxicant

In order to assure that the technical aspects of the bioassay are performed properly, and internal standard toxicant is recommended. The compound we use is sodium dodecyl sulfate (SDS), a surfactant and membrane lytic agent. This compound produces a sharp response curve indicating an almost "all or none" effect. While the use of an internal standard can serve as a quality assurance monitor, it does not, in itself, validate an experiment. Adequate control survival $\geq$85% is the primary criteria for the success or failure of a bioassay.

7. Conclusion

The above culture system, while designed for Acartia tonsa, has worked equally well for Eurytemora affinis and Psuedodiaptimus coronatus. The generation cages, however, were only suitable for A. tonsa because it releases eggs individually. Both E. affinis and P. coronatus produce egg sacs.

In the event that natural sea water is not suitable quality to allow survival, growth, and reproduction of the test species, the following synthetic formulations are recommended. The formulation in Table 1-D has been used for both whole life history culture and numerous bioassay studies at this laboratory. Heinle (1969) found the commercial sea water Instant Ocean suitable for the culture of both A. tonsa and E. affinis. No data is available on the use of Instant Ocean in bioassays and comparisons to natural sea water. In lieu of this, I would only recommend it for culture and not bioassays until suitable comparative data is available.

While it is desirable to work with an age standardized culture this is not always possible. The mass culture system described can be used as a holding and acclimation system for indigenous populations. For example, in many geographical areas A. tonsa is replaced by A. clausi during the winter months. Using the above system we have held A. clausi at 10°C for several weeks. These organisms were used in bioassays with excellent results. Thus we feel that this system, with appropriate modifications, can be used to hold and culture a variety of zooplankters.

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E. STATIC BIOASSAY PROCEDURE USING GRASS SHRIMP (*Palaemonetes* sp.) LARVAE

1. Introduction

Procedures for static 96-hour bioassays utilizing grass shrimp larvae, *Palaemonetes* sp., are outlined here. The grass shrimp is an obvious bioassay choice for several reasons. Three species of the genus, *P. pugio*, *vulgaris*, and *intermedius*, are common inhabitants of estuaries along the Gulf and Atlantic coasts of the United States (Holthuis, 1949, 1952). They are easy to collect and maintain in the laboratory. Field populations are usually quite large, allowing greater numbers to be brought into the laboratory for testing. By manipulating environmental conditions of temperature and photoperiod, it has been possible to induce spawning in the laboratory (Little, 1967), opening the way to laboratory cultures of genetic uniformity. Developing larvae are also available throughout the year for testing with these methods.

Larval stages of the three species are hardy and easy to culture in the laboratory. Developmental stages have been described for all species (Broad, 1957a, b; Broad and Hubschman, 1962; Hubschman and Broad, 1974), and salinity-temperature optima are known for the larval development of *P. vulgaris* (Sandifer, 1973). Developing larvae have demonstrated a susceptibility to polychlorinated hydrocarbons greater than adults or juveniles (Tyler-Schroeder, unpublished manuscript).

2. Culture Methods

Palaemonetes sp. are easily collected from the field using dip nets or seines in grassy, shallow estuarine areas. They can also be reared in enclosed holding ponds.

To obtain larvae, 8" glass culture bowls, such as the Carolina® culture dish, containing 1ℓ of filtered sea water are stocked with 3 ovigerous female shrimp per bowl. In order to produce enough shrimp larvae for a 96-hour test series (210 per replicate, 630 per test series; see Figures 1-E and 2-E) at least 17-25 bowls of ovigerous females (51-75 shrimp) must be maintained continuously in the laboratory. The species of each female is

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Concentration(s) (mg/liter - ppm)	Control	0.01	0.1	1.0	10
Number larvae per test container	30	30	30	30	30
Total $= \frac{\text{150 larvae}}{\text{Replicate}}$					
			1st Day larvae 3 replicates		
Larval age and Number of replicates			18 Day larvae 3 replicates		
	(150 larvae/replicates) X (3 replicates/test) - 450 larvae (450 larvae/test) X (2 test ages) = 900 larvae				
Total number of larvae	Total $= \frac{\text{900 larvae}}{\text{Test Series}}$				
Example mortality:					
%	0	3	10	80	97
ppm	Control	0.01	0.1	1.0	10.0
Estimated LC50 between 0.1 and 1.0 ppm					

Figure 1-E. Example of a range-finding bioassay.

Concentrations (ppm) (chosen from Range-Finding Tests, Figure 1-E)	Control	0.1	0.159	0.252	0.399	0.631	1.0
Number of larvae per test concentration	30	30	30	30	30	30	30
Larval age and number of replicates	1st Day larvae 3 replicates						
	18th Day postlarvae 3 replicates						

Total number (210 larvae/replicate) X (3 replicates/test) = 630 larvae

Larvae (630 larvae) X (3 test ages) = 1890 larvae

$$\text{Total} = \frac{\text{1890 larvae}}{\text{Test Series}}$$

Figure 2-E. Example of a definitive bioassay.

confirmed and the chelipeds removed with fine surgical scissors to prevent removal of the eggs by the females. Shrimp in culture bowls are fed *Artemia* nauplii daily and water is changed if a slight cloudiness appears. Since eggs are carried for 2-3 weeks before hatching; it is advisable to select females with eggs in the more advanced stages of development.

Larvae are removed from bowls containing ovigerous females each morning and mixed together to insure uniformity of test animals. They are randomly dispensed into 8" culture bowls containing 1ℓ of filtered sea water (200 larvae/ℓ), fed *Artemia* nauplii and reared to the desired test age. Food is added daily and water changed when a slight cloudiness appears. There should always be sufficient live food in rearing and test chambers, since insufficient food accentuates developmental variability (Broad, 1957b) and produces undesirable variation in test results.

When rearing larvae to a particular age, a 10-15% mortality should be expected when figuring the number of larvae needed for that test. Ideally, the larvae to be used in a series of 96-hour acute tests should be hatched at one time and reared in mass culture. Samples of larvae would be removed from this culture at designated times for testing. This technique would minimize or circumvent problems due to possible seasonal variation in larval susceptibility to waste material.

Salinity-temperature optima for *P. vulgaris* larvae indicate a broad range of tolerance to environmental conditions, which is most likely true for *P. pugio* and *P. intermedius*. Survival of *P. pugio* is approximately the same when reared in the laboratory at a temperature of 25°C and salinities of from 15-25 $^{o}/oo$ (A.N. Sastry, personal communication[1]). Bioassays should be performed within this range, preferably closer to 15 $^{o}/oo$ salinity, as *P. pugio* taken from the field are most commonly found in water of this salinity, or lower.

3. Preparation of Test Media, Selection of Test Containers

The nature of the material being tested dictates choice of test container size and shape, preparation of test concentrations and frequency of test media replacements. Problems posed by various wastes include insolubility in sea water, adsorption to exposed surfaces, decomposition by hydrolysis, photolysis, etc., loss by volatilization, high BOD and bacterial growth. Such problems can affect results by causing variation from the calculated concentration of waste being tested, changing pH of test medium, releasing breakdown products which may be more or less toxic than parent compounds, and causing mortality of test animals not related to direct effect of toxicants.

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When choosing container size, it is important to choose a small vessel surface area to volume ratio because of possibility of adsorption. A larger volume is also important because of stocking density requirements. The 8" diameter Carolina culture dish, containing 1ℓ of media, has been found to be a satisfactory test container for bioassay of Palaemonetes larvae, allowing maximum volume per vessel surface area and an acceptable stocking density of 30 larvae/ℓ.

Test media should be prepared fresh at the time of replacement, so that decomposition of toxicant, adsorption to preparation containers, depletion of oxygen and bacterial growth is minimal. Likewise, it is necessary to change solutions in test containers at least every 24 hours, preferably every 12 hours.

All sea water to be used should be of natural origin, preferably from the dumping site. It should be filtered through a filter of 1μ porosity. To adjust salinity the addition of either distilled water or a high-salinity brine is necessary. The high-salinity brine may be of natural or artificial origin. If natural origin is desired, place a closed container one-half to three-quarters full of filtered sea water ($\geq$30 °/oo salinity) in a freezer until solid throughout, usually 2-3 days. Subsequent to removal from the freezer, the supernatant is drained after the first 2-3 hours thaw. Supernatant should be 80-110 °/oo salinity or above and can be stored indefinitely.

An artificial brine may be made using any of the commercial artificial sea salts and distilled water, but should be used with caution as several of these preparations contain one or more chelator substances, e.g., EDTA, which would bias test results with waste material containing heavy metals. The use of artificial sea water in place of natural sea water totally, is not recommended at this time. In addition to various chelators in commercial preparation, the presence of high levels of contaminant heavy metals in artificial or laboratory prepared sea salt mixes should be checked. Several shelf chemicals are known to have background levels of Cu, for example, as high as 5-10 ppb (μg/ℓ) (Erickson et al. 1970; J.H. Gentile, personal communication[2]). Unwanted trace metals can be removed by passing the sea water through a column containing a deionizing resin (Davey et al. 1970), but this method may not be practical for large volumes of water.

Many effluents to be tested are complex mixtures having both solid and liquid components. There may also be gaseous components. The following guidelines should be followed when preparing test media:

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A. If Liquid Only

Waste material should be stirred or shaken thoroughly before use. Waste material may be used directly as a stock, or a stock prepared by dilution with filtered sea water to a desired concentration. All stocks and test concentrations should be prepared on a weight to volume basis (gm/ℓ, mg/ℓ, μg/ℓ). If volume/volume basis is used, a correction should be made for specific gravity of the material being tested, i.e., (weight/volume) ÷ (specific gravity) = volume/volume.

B. If Solid and Liquid

1. Agitate the entire material, solid and liquid, until thoroughly mixed. Remove a volume and use as a stock, or dilute with filtered sea water to a desired stock concentration. It will be necessary to provide agitation, e.g., aeration or stirring, in test containers to maintain solid materials in suspension.

2. a) Centrifuge the material to separate solid and liquid fractions. Decant liquid-do not filter. Use as stock or dilute to desired stock concentration as above.

b) It may be desirable to test the effects of solids on Palaemonetes larvae. Solids can be added to the test containers by weight, agitated to keep them in suspension and combined toxic-mechanical effects determined. Alternately, one volume of solid material may be diluted with four volumes of sea water to prepare a standard elutriate. The "standard elutriate" is the supernatant resulting from the vigorous 30 minute shaking of one part bottom sediment with four parts water from the proposed disposal site followed by 1 hour of letting the mixture settle and appropriate filtration or centrifugation (Federal Register 38(94):12874, Wednesday, May 16, 1973). Use the standard elutriate as a stock and assay.

Complex wastes often contain substances insoluble in sea water, necessitating the use of a water miscible solvent to introduce them into the test media. Only the minimum amount of solvent necessary to dissolve the toxicant should be used, preferably no more than 100 μl solvent/ℓ of test media and only 10 μl/ℓ, if possible. Test concentrations should be prepared so that the amount of solvent added to each test container is constant. A solvent control must be run with each test to show the solvent by itself exerts no adverse effects on test organisms. Suggested solvent carriers are polyethylene glycol (M.W. 200), triethylene glycol, acetone and ethanol.

All test glassware should be thoroughly washed using the following procedure:

1) Empty old test solution and rinse with cold water.

2) Rinse with acetone, followed by a warm water rinse.

3) Wash with laboratory soap and a brush. Rinse thoroughly with warm water 4-5 times.

4) A rinse of 10% HCl or HNO_3 is required also, if the toxicant contains heavy metals.

5) Rinse with distilled water and allow to dry. If an acid rinse is used, it should be followed by 4-5 thorough rinses with deionized water.

4. Bioassay Procedures

Because Palaemonetes normally exhibits variability in molting and developmental rates during larval life, it is not feasible to produce sufficient larvae of individual stages for testing. Therefore, tests are begun using larvae of specified ages (e.g., ages 1, and 18 days). Most larvae will metamorphose to postlarvae (PL) on approximately day 18 - 21. Hence, one bioassay is performed on 18 day old larvae and one on postlarvae, to determine if the biochemical and physiological changes accompanying metamorphosis alter the response to the toxicant. For the same reasons, a bioassay using day 30 postlarvae may be required.

Palaemonetes larvae are added to test containers using a method of random selection (total randomization, stratified randomization, etc.). Larvae are removed from culture dishes using a rectangular piece of fine mesh Nitex nylon net and stocked in test dishes at a density of 30 larvae/liter of test media/culture dish. Larvae are fed an excess supply of Artemia nauplii throughout the test. Artemia are added with each change of test media. Mortalities of Palaemonetes are recorded at the time of each test media change (every 12 or 24 hours), and all dead animals removed at this time. All test and control culture dishes should be maintained at 25°C in a culture cabinet, BOD incubator, or water table. Tests may be run in total darkness or on a 12 hr light - 12 hr dark regime. All tests should include 4-6 concentrations and a sea water control. Control mortality exceeding 10% invalidates test results. Because of the inherent variability of each age group of larvae, 2 to 3 replicates must be run simultaneously for each test concentration in each experiment. These basic test conditions are to be followed for both range-finding and definitive bioassays as discussed below (See Figures 1-E and 2-E).

Initially, a series of range-finding 96-hour assays are performed using 1 and 18 day old larvae to determine the range of toxicity of the material being examined, and to determine the best test conditions. A broad range of concentrations covering at least four orders of magnitude should be tested, e.g., 0.01, 0.1, 1.0 and 10 mg/ℓ (ppm), or gm/ℓ (%). Temperature, pH and dissolved oxygen (DO) levels should be monitored throughout these tests to help determine need for aeration and frequency of test solution change.

After the range-finding test is completed and an LC50, concentration lethal to 50% of the shrimp, approximated, a series of definitive bioassays are performed. The purpose of the definitive bioassay is to more clearly determine the limits of toxicity of a waste and better estimate the LC50.

Concentrations chosen for definitive bioassays are determined by results from the range-finding tests, i.e., the lowest definitive test concentration should equal or be greater than the greatest concentration in range-finding tests that killed none or few test organisms. Likewise, the greatest definitive test concentration should be equal to or less than the least concentration in range-finding tests that killed all or almost all of the test organisms (See Figures 1-E and 2-E). Once upper and lower definitive test concentrations are chosen, intermediate concentrations are calculated using progressive bisection of intervals on a logarithmic scale (Standard Methods, 1965). At least five, and preferably more, test concentrations are to be used in order to yield mortality data that lies on either side of a 50% kill, a condition necessary for statistical treatment of data using Probit Analysis.

Growth is often a more sensitive indication of effect than mortality and is useful in choosing concentrations to be used for chronic tests. Therefore, at the end of each test the rostrum-telson length of surviving larvae from each test concentration and controls should be measured using an ocular micrometer. A sample of untreated larvae should be measured at the beginning of the test for comparative purposes. Additional observations, such as loss of equilibrium, cessation of feeding, irregular movements, and other behavioral aberrations should be noted at the time of each test media change.

5. Analysis of Data

Data from 96-hour acute bioassays should be analyzed using Probit Analysis (Finney, 1964a, b). This method estimates a value for LC30, 70 and 90, as well as the LC50. Because Probit Analysis is generally performed by computer, it is wise to check the computer output, by plotting percent kill in probits against logarithm of concentration and comparing computer and graphed LC50's. The line thus plotted should closely resemble that determined by the computer. The Litchfield-Wilcoxon method of LC50 estimation or graphical interpolation using Probit graph paper (Standard Methods, 1965) can be used when data does not meet the more rigorous specifications required by Probit Analysis (Litchfield, 1949; Litchfield and Wilcoxon, 1949, 1953). The 95% confidence limits should be indicated for all data.

6. Reports

At the completion of testing and data analysis, a report is usually required. Such reports should include the following information:

1. Name of method, investigator, laboratory, and date tests were conducted.

2. Detailed description of material tested, source, date and time of collection, composition, known physical and chemical properties.

3. Source of sea water, date and method or preparation.

4. Detailed information about test animals, including scientific name, life stage, age, source, history and acclimation procedure for larvae, if necessary.

5. A description of experimental design, test containers, volume of test solution, way test was begun, number of organisms at each concentration, number of organisms in each control and types of controls run.

6. Definitions of response used to determine the effect and a summary of general observations of other effects or symptoms.

7. Percent of control organisms that died or were affected during the test.

8. LC50, with confidence limits. LC30, 70 and 90, if pertinent.

9. Methods used for and results of all DO, pH, and temperature measurements.

10. Any deviations and reasons.

11. Other relevant information.

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F. STATIC METHOD FOR ACUTE TOXICITY TESTS USING FISH AND MACROINVERTEBRATES

1. Equipment

a. Facilities

For maximum convenience and versatility, the facilities should include tanks or live cars for holding and acclimating test animals, a tank for salt water, and a temperature-controlled recirculating water bath or controlled-environment room for the test containers. The holding and acclimation tanks should be equipped for temperature control and the holding tank should be equipped for aeration. Because air used for aeration must not contain oil or fumes, it must be taken from a well-ventilated, fume-free area and powered by a surface aerator or an oil-less rotary or piston-type air compressor. During holding, acclimation, and testing, test animals should be shielded from disturbances.

b. Construction Materials

Construction materials and commerically purchased equipment that may contact any water into which test animals are to be placed, should not contain any substances that can be leached or dissolved by the water. In addition, materials and equipment should be chosen to minimize sorption of toxicants from water. It is suggested that glass, #316 stainless steel, or perfluorocarbon plastics be used whenever possible.

c. Test Containers

1. Type: For fish and invertebrates, the test solution should be between 15 and 20 centimeters (cm) deep. These animals can be tested in 19.6ℓ (5-gallon) wide-mouthed soft glass bottles containing 15ℓ of solution. Alternatively, test containers can be made by welding, not soldering, stainless steel or by gluing double-strength window glass with clear silicon adhesive. As little adhesive as possible should be in contact with the water; extra beads of adhesive should be on the outside of the containers rather than on the inside. Some invertebrates can be tested in 3.9ℓ (1 gallon) wide-mouthed soft glass bottles or battery jars.

2. Cleaning: Test containers must be cleaned before use. New containers must be washed with detergent and rinsed with 10% hydrochloric acid, acetone, and tap or other clean water. At the end of every test, if the test containers are to be used again, they should be (1) emptied; (2) rinsed with water; (3) cleaned by a procedure appropriate for removing the toxicant test, e.g., acid to remove metals and bases; detergent, organic

solvent, or activated charcoal to remove organic compounds; and (4) rinsed with water. Acid is useful for removing scale and hypochlorite (bleach) is useful for removing organic matter and for disinfecting. All test containers must be rinsed with salt water just before use.

2. Salt Water

For acute toxicity tests, a practical criterion for an acceptable salt water is that healthy test animals will survive in it for the duration of acclimation and testing without showing signs of stress, such as unusual behavior or coloration. Salt water should be prepared from commercially available formulations or from ingredients listed in Table 1-F using deionized or glass-distilled water. Deionized or distilled water should be used to dilute the salt water to a salinity of 30 parts per thousand (°/oo). Natural salt water may also be used if it satisfies the acclimation requirement.

TABLE 1-F. STANDARD SALT WATER*

Ingredient	Amount (g)	Ingredient	Amount (g)
$SrCl_2.6H_2O$	0.02	$MgCl_2.6H_2O$	10.0
H_3BO_3	0.03	NaCl	23.50
KBr	0.10	$Na_2SiO_3.9H_2O$	0.02
$CaCl_2.2H_2O$	1.10	EDTA†	0.003
Na_2SO_4	4.00		

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†Ethylene diamine tetracetate.

*To formulate this water, mix technical grade salts with 900 mℓ of distilled or demineralized water in the order and quantities listed. Then add enough distilled or demineralized water to make the final volume 1ℓ . Dilute the water with distilled or demineralized water to achieve a salinity of 30 °/oo. if necessary, add NaHCO to adjust final pH of water to between 8.0 and 8.2. Before the water is used, filter it through a 0.22-micron membrane filter.

3. Test Organisms

a. Species

Recommended test animals are as follows (specific names must be verified and reported):

Invertebrates:

White sea urchin, Tripneustes esculentus
White shrimp, Penaeus setiferus
Pink shrimp, P. duorarum
Brown shrimp, P. aztecus
Grass shrimp, Palaemonetes sp.
Shrimp, Crangon sp.
Oceanic shrimp, Pandalus jordani
Blue crab, Callinectes sapidus
Dungeness crab, Cancer magister

Vertebrates:

Sheepshead minnow, Cyprinodon variegatus
Mummichog, Fundulus heteroclitus
Silverside, Menidia sp.
Threespine stickleback, Gasterosteus aculeatus
Pinfish, Lagodon rhomboides
Spot, Leiostomus xanthurus
Shiner perch, Cymatogaster aggregata
Buffalo sculpin, Enophrys bison
Pacific staghorn sculpin, Leptocottus armatus
English sole, Parophrys vetulus

Other species indigenous to the dumping area may be used if approved by EPA. The specific name of the animals must be verified and reported. Samples of the test animals may be requested by EPA. Tests on other animals under other experimental conditions may be required by EPA.

b. Source

Test animals are usually collected from wild populations in relatively unpolluted areas. (Collecting permits may be required by local or state agencies.) Some animals may be purchased from commercial suppliers. All animals should be healthy and as uniform in size and age as possible.

c. Size

1. Fish: Fish that weigh between 0.5 and 5.0 grams each are usually desirable. In any single test, the standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than two times the standard length of the shortest fish.

2. Invertebrates: Maximum size should be:

shrimp - less than 10 cm rostrum-telson length
crabs - less than 10 cm carapace width

Since cannibalism occurs in many species of arthropods, the claws of crabs should be banded, or the individuals should be physically isolated.

d. Care and handling

If the animals are to be tested at a temperature or salinity other than that at which they are collected, they should not be subjected to more than a 3°C change in water temperature in any one hour period or to more than a 5 °/oo change in salinity in any 24 hour period. To maintain animals in good condition during holding and acclimation, crowding should be avoided. Animals should be fed at least once a day if held for an extended period and tanks should be cleaned after feeding.

Animals should be handled as little as possible. When they must be handled, it should be done as gently, carefully and quickly as possible.

A group of organisms must not be used for a test if they appear to be diseased or otherwise stressed or if more than 3% of the individuals die during the 48 hours immediately prior to transferral to test containers.

4. Recommended Procedure for Testing Material

a. Experimental Design

The recommended test procedure consists of two separate 96-hour bioassays with a control and six concentrations of the material to be tested. One 96-hour bioassay will be without aeration and the second will be with aeration. In the latter, containers will be aerated with clean air at the rate of 100 ± 15 bubbles/minute delivered from a glass tube 1 mm inside diameter. (Use of several disposal pipetts connected to a side-arm vacuum bottle provides for easier control of air flow.)

There must be at least 10 control animals and at least 10 animals must be exposed to each concentration or dilution of the material to be tested, but they may be divided between two or more containers. The use of more animals and replication of treatments is desirable. If replicates are used, they should be true replicates with no water connection between the replicate test containers. Stratified randomization of the treatments (random assignment of one test container for each treatment in a row followed by random assignment of a second test container for each treatment in another or an extension of the same row) or total randomization of the treatments is recommended. A representative sample of the test animals should be impartially distributed to the test containers, either by adding one (if there are to be less than 11 animals per container) or two (if there are to be more than 11 animals per container) test animals to each container, and then adding one or two more to each test container, and repeating the process until each test container has the desired number of test animals in it. Alternatively, the animals can be assigned either by total randomization or by stratified randomization (random assignment of one animal to each test container, random assignment of a second animal to each test container, etc.).

Every test requires a control which consists of the same salt water, conditions and animals as are used in containers with test material. A test is not acceptable if more than 10% of the control animals die.

b. Temperature

Test water temperature must be maintained within 1°C of the water temperature listed in Table 2-F.

TABLE 2-F. SUGGESTED TEST TEMPERATURES FOR VERTEBRATES AND INVERTEBRATES*

Region	Temperature
I	20°C
II† and III	25°C
IV, VI and IX	30°C
X	15°C

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*Temperatures in this table should be revised to the highest average monthly temperature of oceanic surface waters at dump sites in each region.

†Puerto Rico and Virgin Islands are in Region II but should use temperatures suggested for Region IV.

c. Salinity Test

Test water salinity should be 30 °/oo before the material to be tested is added.

d. Loading

The mass of animals in each test container must be limited so that the animal's oxygen requirements alone do not influence the test results. For the recommended test animals, the grams of animals per liter of test solution in the test containers should not exceed 1.0. Tests at high temperature may require reduced loading. Proper loading can be confirmed by measuring dissolved oxygen concentration in the water of the unaerated control containers. It must not be less than 4 mg/ℓ (ppm).

e. Preparation of Material to be Tested (See other section of manual on this subject)

Samples, whether liquid waste or sludge, will be stirred to a uniform consistency before dilutions are made.

f. Concentrations

Dilutions of samples, by volume, of 10% (100,000 ppm, 100 ml/ℓ), 1% (10,000 ppm, 10 ml/ℓ), 0.1% (1,000 ppm, 1 ml/ℓ), 0.01% (100 ppm, 0.1 ml/ℓ), 0.001% (10 ppm, .01 ml/ℓ), and 0.0001% (1 ppm, 0.001 ml/ℓ) are recommended as initial test concentrations.

The highest concentration (dilution) will be prepared as follows: 9 volumes of salt water will be added to 1 volume of the stirred sample. (Adequate space should be reserved in the test container for stirring and addition of animals.)

Each succeeding concentration will be prepared by a similar 1-in-10 serial dilution from the previous test container. Adequate stirring of the contents of the test container is essential before each dilution.

g. Aeration of One Bioassay

Following dilution, aeration of one set of concentrations should begin using 100 ± 15 bubbles per minute delivered through a 1 mm ID glass tube.

h. Transfer of Animals

Animals must be added to the test containers within 1 hour after the proper dilutions of the material to be tested have been made and aeration of one set of test containers begun.

i. Feeding

The organisms must not be fed while in the test containers.

j. Measurements

The dissolved oxygen concentration, pH, and temperature must be measured (1) before adding animals and (2) at 24-hour intervals thereafter in the highest and lowest concentration and the control of both aerated and unaerated bioassays. Additional measurements are required in containers in which animals die. Water samples should be taken midway between the top, bottom, and sides of the test containers and should not include any surface scum of material stirred up from the bottom or sides.

k. Observations

At a minimum, the number of dead or affected animals must be recorded at 24-hour intervals throughout the test. More observations are often desirable, especially near the beginning of the test. Dead animals must be removed as soon as they are observed.

The adverse effects most often used to study acute toxicity with aquatic animals is death. Criteria for death are no movements, especially no opercular movement in fish, and no reaction to gentle prodding. However, death is not easily determined for some invertebrates, and so an EC50 (effective concentration to 50% of test animals) is often measured rather than an LC50 (lethal concentration to 50% of test animals). The effect generally used for determining an EC50 with invertebrates is immobilization which is defined as the inability to move except for minor activity of appendages, or loss of equilibrium. Other effects can be used for determining an EC50, but the effect and its definition must always be reported. General observations on such things as erratic swimming, loss of reflex, discoloration, changes in behavior, excessive mucous production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism should be reported.

5. Calculation and Reporting

At the end of the test period, the bioassays are terminated and the LC50 or EC50 values are determined.

a. Calculations

An LC50 is a concentration at which 50% of the experimental animals died and an EC50 is a concentration at which 50% of the experimental animals were affected. Either may be an interpolated value based on percentages of animals dying or affected at two or more concentrations. Estimating the LC50 or EC50 by interpolation involves plotting the data on semilogarithmic coordinate paper with concentrations on the logarithmic axis and percentages of dead or affected animals on the arithmetic axis. A straight line is drawn between two points representing death or effect in concentrations that were lethal to or effective against more than half and less than half of the organisms. The concentration at which the line crosses the 50% mortality or effect line is the LC50 or EC50 value. If 50% of the test animals are not affected by the highest concentration, the percent affected should be reported.

b. Reports

Any deviation from this method must be noted in all reports of results. A report of the results of both aerated and unaerated tests must include:

1. name of method, author, laboratory, and date tests were conducted;

2. a detailed description of the material tested, including its source, date and time of collection, composition, known physical and chemical properties, and variability of the material tested;

3. the source of the salt water, date prepared and method of preparation;

4. detailed information about the test animals, including name, standard length, weight, source, history, and acclimation procedure used;

5. a description of the experimental design, the test containers, the volume of test solution, the way the test was begun, the number of organisms per concentration, and the loading;

6. definitions of the criteria used to determine the effect and a summary of general observations on other effects or symptoms;

7. percent of control organisms that died or were affected in each test container;

8. the 24-, 48- and 96-hour LC50, or EC50;

9. methods used for and the results of all dissolved oxygen, pH and temperature measurements; and

10. any other relevant information.

G. CONTINUOUS - FLOW METHOD FOR ACUTE TOXICITY TESTS USING FISH AND MACROINVERTEBRATES

1. Introduction

Continuous-flow (often referred to as "flow-through") bioassays have definite advantages over static tests in evaluating certain types of wastes to be disposed of at sea. They are desirable in testing waste chemicals that have high biochemical oxygen demands, and are unstable or volatile. Many test species of fish and macroinvertebrates have high rates of metabolism and are difficult to maintain in jars or tanks of standing sea water. Continuous-flow bioassays, conducted under proper conditions, provide for well-oxygenated test solutions, nonfluctuating concentrations of the toxicant, and continual removal of metabolic wastes of the test organisms. (Standard Methods, 13th Edition, 1971).

This method provides general procedures for conducting a 96-hour, flow-through bioassay on marine fish and macroinvertebrates such as shrimp and crabs. Evaluation of different types of waste will, no doubt, require some modification of these procedures.

2. Equipment

a. Facilities

For maximum convenience and versatility, the facilities should include tanks or live cars for holding and acclimating test animals, a tank for sea water, and a temperature-controlled recirculating water bath or controlled-environment room for the test containers. The holding and acclimation tanks should be equipped for temperature control and the holding tank should be equipped for aeration for emergency use. During holding, acclimation, and testing, test animals should be shielded from unnecessary disturbances.

b. Construction Materials

Construction materials and commercially purchased equipment that may contact any water into which test animals are to be placed should not contain any toxic substances that can be leached or dissolved by the water.

In addition, materials and equipment should be chosen to minimize sorption of toxicants from water. It is suggested that glass, #316 stainless steel, or perfluorocarbon plastics be used whenever possible.

c. Test Containers

1. Type: For fish and invertebrates, the test solution usually needs to be between 15 and 20 cm deep. Test containers can be made by welding, not soldering, stainless steel or by gluing double-strength window glass with clear silicon adhesive. As little adhesive as possible should be in contact with the water; extra beads of adhesive should be on the outside of the containers rather than on the inside. Plywood tanks coated with fiberglass resin are also acceptable.

2. Cleaning: Test containers must be cleaned before use. New containers must be washed with detergent and rinsed with 10% hydrochloric acid, acetone, and tap or other clean water. At the end of every test, if the test containers are to be used again, they should be (1) emptied; (2) rinsed with water; (3) cleaned by a procedure appropriate for removing the toxicant tested, e.g., acid to remove metals and bases; detergent, organic solvent, or activated charcoal to remove organic compounds; and (4) rinsed with water. Acid is also useful for removing scale and hypochlorite (bleach) is useful for removing organic matter and for disinfecting. All test containers must be rinsed with uncontaminated sea water just before use.

3. Sea Water

For acute toxicity tests, a practical criterion for an acceptable sea water is that healthy test animals will survive in it for the duration of acclimation and testing without showing signs of stress, such as unusual behavior or coloration. Natural sea water (particularly from the dump site) is preferable to artificial sea water; however, at times due to logistical or economical problems, artificial sea water s more prctical. Artificial sea water may be prepared from commercially available formulations or from ingredients listed in Table 1-G using deionized or glass-distilled water. Salinity of test water should ideally be that of the dump site; however, requirements of the individual species to be tested must be considered.

TABLE 1-G. ARTIFICIAL SEA WATER*

INGREDIENT	AMOUNT (g)	INGREDIENT	AMOUNT (g)
$SrCl_2.6H_2O$	0.02	$MgCl_2.6H_2O$	10.00
H_3BO	0.03	NaCl	23.50
KBr	0.10	$Na_2SiO_3.0H_2O$	0.02
$CaCl_2.2H_2O$	1.10	EDTA†	0.003
Na_2SO_4	4.00		

†Ethylene diamine tetracetate (EDTA)

*To formulate this water, mix technical grade salts with 900 milliliters of distilled or demineralized water in the order and quantities listed. Then add enough distilled or demineralized water to make the final volume one liter. Dilute the water with distilled or demineralized water to achieve a salinity of 30 ^{o}/oo. If necessary, add NaHCO to adjust final pH of water to between 8.0 and 8.2. Before the water is used, filter it through a 0.22 micron membrane filter.

4. Test Organisms

a. Species

Recommended species are as follows (specific name must be verified and reported):

Invertebrates:

Copepods, *Acartia* sp.
White sea urchin, *Tripneustes esculentus*
White shrimp, *Penaeus setiferus*
Pink shrimp, *P. duorarum*
Brown shrimp, *P. aztecus*
Grass shrimp, *Palaemonetes* sp.
Shrimp, *Crangon* sp.
Oceanic shrimp, *Pandalus jordani*
Blue crab, *Callinectes sapidus*
Dungeness crab, *Cancer magister*

Vertebrates:

Sheepshead minnow, *Cyprinodon variegatus*
Mummichog, *Fundulus heteroclitus*

Longnose killifish, *F. similis*
Silverside, *Menidia* sp.
Threespine stickleback, *Gasterosteus aculeatus*
Pinfish, *Lagodon rhomboides*
Spot, *Leiostomus xanthurus*
Shiner perch, *Cymatogaster aggregata*
Buffalo sculpin, *Enophrys bison*
Pacific staghorn sculpin, *Leptocottus armatus*
English sole, *Parophrys vetulus*

Other species indigenous to the dumping area may be used if approved by EPA and the specific name of the organism is verified and reported. Samples of the test animals may be requested by EPA. Tests on other organisms under other experimental conditions may be required by EPA.

b. Source

Test animals are usually collected from wild populations in relatively unpolluted areas. (Collecting permits may be required by local or state agencies.) Some animals may be purchased from commercial suppliers. All animals should be healthy and as uniform in size and age as possible. Juvenile stages are preferable.

c. Size

1. Fish: Fish that weigh between 0.5 and 5.0 g each are usually desirable. In any single test, the standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than two times the standard length of the shortest fish.

2. Invertebrates: Maximum size should be:

shrimp - 5 to 10 cm rostrum-telson length (5-8 g live weight)
crabs - less than 7 cm carapace width

d. Acclimation

Depending on the desired test conditions, different periods of acclimation may be necessary. Acclimation to ambient laboratory conditions should be the most severe stress the animals will encounter (before the actual test) i.e., handling in the field and transportation to the laboratory with the inevitable shock of placing the animals in water different than that of the transporting media. Initial transfer of the animals in the lab should be made to water with the temperature and salinity adjusted very closely to those conditions found in the transporting media. Acclimation to ambient laboratory conditions should be considered successful if less than 10% of the animals die during 4-7 days of holding. If the exposure test is to be run at controlled conditions of temperature and salinity other than ambient, the test animals should be proportioned into the exposure tanks containing water at ambient conditions. The water conditions can then be changed gradually (8-24 hours) to the test conditions if temperature changes less than +3°C or -5°C and salinity changes less than 5 ^{o}/oo. Under these conditions acclimation without handling should continue for 4-10 days with adequate water flow.

e. Care and Handling

Animals should be handled as little as possible. When they must be handled, it should be done with a dip net as gently, carefully, and quickly as possible. Animals should be fed daily during acclimation but should not be fed for a period of 48 hours before or during the actual test. It may be necessary, however, to feed certain invertebrates during the actual test.

Crowding should be avoided. Cannibalism occurs in many species of arthropods; therefore, in some cases it may be necessary to isolate individuals in compartmented aquaria. Banding the claws of crabs, and placing a 2-3 cm (about 1 in.) layer of sand in the bottom of the aquaria for shrimp are useful techniques.

5. Recommended Procedure for Testing Material

a. Experimental Design

The recommended test procedure consists of a 96-hour bioassay with a control and at least five concentrations of the material to be tested. Acute static tests are useful in determining range of toxicity of the material and selecting concentrations for the flow-through tests (See Section 5-f. Range finding and definitive tests).

For the definitive test a minimum of 20 organisms is required for the control and each concentration or dilution of the material to be tested, but they may be divided between two or more test containers. The use of more organisms and replication of treatments is desirable but consideration of "loading" must be made. If replicates are used, they should be true replicates with no water connection between the replicate test containers. Stratified randomization of the treatments (random assignment of one test container for each treatment in a row followed by random assignment of a second test container for each treatment in another or an extension of the same row) or total randomization of the treatments is recommended.

The test animals should be impartially distributed to the test containers by adding no more than 10% to each container, repeating the process until each test container has the desired number of test animals in it. Alternatively, the animals can be assigned either by total randomization or by stratified randomization (random assignment of one animal to each test container, random assignment of a second animal to each test container, etc.).

Every test requires a control which consists of the same salt water, conditions and animals of same species and size as are used in containers with test material. A test is not acceptable if more than 10% of the control animals die.

b. Toxicant Delivery System

Flowing sea water tests are preferable to static tests because test solutions are renewed continually, assuring a steady concentration of the toxicant, however, they require metering pumps or other devices for accurate delivery of the toxicant or test material into the sea water flowing through the test aquaria.

Most toxicant delivery systems have been designed for introducing solutions of toxicants and solvents into fresh water, and may not be applicable to all wastes. Many materials proposed for disposal at sea are not homogenous mixtures and innovative toxicant delivery systems will be required to introduce representative samples of the materials into the test containers. Stirring may be required to maintain suspended solids in non-homogenous dump material.

Many toxicant delivery systems have been described and used in various types of bioassay (Sprague, 1969; Freeman, 1971; Bengtsson, 1972; Cline and Post, 1972; Granmo and Kollberg, 1972; Lowe et al. 1971 and 1972; Lichatowich et al. 1973; Abram, 1973), but the proportional diluter (Mount and Brungs, 1967) has probably been used routinely (in fresh water) more than any other system. A small chamber to promote mixing of toxicant-bearing water and dilution water should be used between the diluter and the test containers for each concentration. If duplicate test containers are used, separate delivery tubes can be run from this mixing chamber to each duplicate. Alterations in the design of the proportional diluter, have been useful in some situations (Esvelt and Conners, 1971; McAllister, Mauch and Mayer, 1972; Benoit and Pulglisi, 1973; Schimmel, Hansen and Forester, 1974).

The flow rates through the test containers must be at least 5 tank water volumes per 24 hours, and in many cases it is desirable to construct the toxicant delivery system so that it can provide 10 or more tank water volumes per 24 hours. Some systems may provide for a continuous flow of sea water. The flow rates through the test containers should not vary by more than 10% from any one test container to any other or from one time to another within a given test.

The calibration of the toxicant delivery system should be checked carefully before, during and after each test. This should include determining the volume of stock solution and dilution water used in each portion of the toxicant delivery system and the flow rate through each test container. The general operation of the toxicant delivery systems should be checked daily during the test.

c. Temperature

Test water temperature should be maintained within 1°C of the water temperature listed in Table 2-G (unless seasonal bioassays are performed). This may be accomplished by preheating the sea water before it enters the test containers, by immersing the test containers in a constant temperature water bath, or by a combination of these methods.

TABLE 2-G. SUGGESTED TEST TEMPERATURES FOR VERTEBRATES AND INVERTEBRATES[*]

Region	Temperature
I	20°C
II[†] and III	25°C
IV, VI and IX	30°C
X	15°C

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[*] Temperature in this table should be revised to the highest average monthly temperature of oceanic surface waters at dump sites in each region.

[†] Puerto Rico and Virgin Islands are in Region II but should use temperatures suggested for Region IV.

d. Salinity

The salinity of test water should be that of the dump site if: (a) dump site water is used or (b) artificial sea water is prepared. The salinity of any other natural sea water used should be $\geq$15 ^{o}/oo.

e. Loading

Excessive weight (grams/liter) of organisms in a test container may adversely affect results of test. Therefore, the loading must be limited so that:

1. the concentration of dissolved oxygen in the control container does not fall below 60% saturation;

2. the concentration of metabolic products does not become too high, specifically, the concentration of non-ionized ammonia does not exceed 20 µg/ℓ;

3. the concentration of toxicant is not lowered by more than 20% because of uptake by the test organisms; and

4. the organisms are not stressed because of crowding - for the species listed under 4. (Test Organisms) the loading in the test containers must not exceed 2 g/ℓ/day. Lower loadings must be used if necessary to meet the four criteria listed above.

f. Range-Finding and Definitive Tests

Much time and effort may be saved by conducting a series of "range-finding", static tests with a few animals and a wide range of concentrations, before setting up the "definitive" flow-through tests which will be used to calculate the final LC50 or EC50 (See Standard Methods, 13th Edition, 1971 for details). For example, waste concentrations of 10, 1, 0.1, and 0.01% by volume and two or three animals in each concentration might be tested first for a period of 24 hours. Definitive test concentrations should then fall between the highest concentration at which all animals survive and the lowest concentration at which all or most animals die.

g. Observations

At a minimum, the number of dead or affected animals must be recorded at 24 hour intervals throughout the test. More observations are often desirable, especially near the beginning of the test. Dead animals must be removed as soon as they are observed.

The adverse effect most often used to study acute toxicity with aquatic animals is death. Criteria for death are no movements, especially no opercular movement in fish, and no reaction to gentle prodding. However, death is not easily determined for some invertebrates, and so an EC50 (effective concentration to 50% of test animals) is often measured rather than an LC50 (lethal concentration to 50% of test animals). The effect generally used for determining an EC50 with invertebrates is immobilization, which is defined as the inability to move except for minor activity of appendages, or loss of equilibrium. Other effects can be used for determining an EC50, but the effect and its definition must always be reported. General observations on such things as erratic swimming, loss of reflex, discoloration, changes in behavior, excessive mucous production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism should be reported.

6. Calculations and Reporting

At the end of the test period, the bioassays are terminated and the LC50 or EC50 values are determined.

a. Calculations

An LC50 is a concentration at which 50% of the experimental animals would be expected to die and an EC50 is a concentration at which 50% of the experimental animals would be expected to be affected. Either may be an interpolated value based on percentages of animals dying or affected at two or more concentrations. Estimating the LC50 or EC50 by interpolation involves plotting the data on logarithmic - probability graph paper with concentrations on the logarithmic axis and percentage of dead or affected animals on the probability axis. A line is drawn between all data points. The concentration which the line crosses the 50% mortality or effect line is the LC50 or EC50 value. In fitting, the line points nearest the 50% effect level should be given more weight. Ideally data should consist of enough intermediate (between 0 and 100%) effects to conduct statistical tests such as probit analysis to determine confidence limits.

If 50% of the test animals are not affected by the highest concentration, the percent affected at each concentration must be reported.

b. Reports

The final report should include:

1. name of method, author, laboratory, and date tests were conducted;

2. a detailed description of the material tested, including its source, date and time of collection, composition, known physical and chemical properties, and variability of the material tested;

3. the source of the salt water, date prepared and method of preparation;

4. detailed information about the test animals, including name, standard length of fishes, carapace width of crabs, total length of shrimp, weight, source, history, and acclimation procedure used;

5. a description of the experimental design, the test containers, the volume of test solution, the number of organisms per concentration, and the loading (water flow to each tank);

6. definitions of the criteria used to determine the effect and a summary of general observations on other effects or symptoms;

7. percent of control organisms that died or were affected in each test container;

8. the 24-, 48-, and 96-hour LC50's or EC50's;

9. methods used for the results of all dissolved oxygen, pH, and temperature measurements; and

10. any other relevant information.

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SECTION III

SPECIAL BIOASSAY PROCEDURES

A. FLOWING SEA WATER TOXICITY TEST USING OYSTERS (Crassostrea virginica)

The following test procedure is included as a "special bioassay" for use in evaluating short-term effects of specific wastes on marine molluscs. It is recommended only for use with the commercial Eastern oyster, Crassostrea virginica, and requires flowing unfiltered, natural sea water. This test should be used only with materials which can be dissolved in water or other solvents and then metered into test aquaria. The test has proved to be a valuable bioassay procedure at the Gulf Breeze Environmental Research Laboratory (EPA) where it has been used for several years to evaluate the effects of insecticides, herbicides, and other toxic organics on oysters (Butler, 1965).

This procedure, as described below, was taken almost verbatim from a report by the Subcommittee on Mollusks of the Standard Bioassay Committee for the 14th Edition of Standard Methods for the Analysis of Water and Waste Water. It is included in this manual by permission of Dr. Philip A. Butler, Chairman of the Subcommittee.

1. Shell Deposition Test

The deposition of new shell in juvenile oysters is directly affected by changes in ambient water quality. The degree of inhibition in shell deposition is quantitatively related to the amount of environmental stress.

This 96-hour test demonstrates the comparative toxicity of pollutants to young oysters. The test is conducted with flowing unfiltered sea water in the temperature range between 15° and 30°C. Actively feeding oysters extend their mantle edges to the periphery of the shell or valves. The body can contract, however, to occupy a much smaller area. If the peripheral valve edges are mechanically ground away, the oysters respond by depositing new shell to replace this loss.

The growth of new shell is primarily linear during the first week and the rate of deposition is an index of the animal's reaction to ambient water quality. With acceptable water conditions, 25 mm and larger oysters deposit as much as 1.0 mm of peripheral new shell per day. Small oysters (less than 50 mm) are more suitable than large ones because typically they form new shell deposits at temperatures ranging from about 10° to 30°C in contrast to mature oysters, which tend to become less active at temperature extremes.

Test data are independent of minor fluctuations in temperature and salinity during the 96-hour exposure, since the simultaneous shell deposition in control oysters is considered to be the norm or 100%.

a. Procurement and Preparation of Oysters

Oysters, about 25 to 50 mm in height, i.e., the long axis, with reasonably flat, rounded shape are culled to singles, cleaned and maintained in trays in the natural environment. At the time of the test, oysters are re-cleaned and about 3-5 mm of the shell periphery are removed, leaving a smoothly rounded blunt profile. This is conveniently done by hand-holding the oysters against an electric disc grinder. Removal of too wide a rim of shell will make an opening into the shell cavity and such damaged oysters should be discarded.

Test aquaria may be fabricated of glass or fiberglassed wood, and should measure about 64 x 38 x 10 cm deep (25 x 15 x 4 inches) to provide adequate space for 20 oysters. Such containers permit adequate circulation of the water while avoiding physical agitation of the oysters by the water current.

The unfiltered water supply in a constant head reservoir is delivered by calibrated siphons to the aquaria via a mixing trough into which the toxicant in an appropriate solvent is also metered. Stock solutions of the toxicant are prepared so that a delivery of 1 or 2 ml per minute by means of a calibrated pump will result in the desired concentration. Baffles in the trough ensure adequate mixing and aeration before the water enters the test aquaria.

The aquaria contain about 18ℓ at 75% capacity and with a flow rate of 100ℓ hour^{-1} will provide 5ℓ of water hour^{-1} oyster^{-1}. Small oysters feed and grow readily under these conditions.

b. Bioassay Procedure

Oysters are prepared and randomly distributed so that each control and test aquarium contains 20 individuals. Oysters are placed with the left, cupped value down and the anterior hinged ends all oriented in one direction. One control aquarium receiving toxicant solvent alone and one aquarium for each desired concentration of the toxicant are established.

At the end of 96 hours, all oysters are removed from the water and the shell increments are measured. Shell deposition is not uniform on the periphery and so the length of the longest "finger" of new shell on each oyster, measured to the nearest 0.5 mm, is recorded.

c. Calculation

The ratio of the mean growth of a group of test oysters to the mean growth of the control oysters provides a percentage index of the tolerance of the oysters to a specified toxicant concentration. A 96-hour EC50 (concentration inhibiting shell deposition by 50%) may be calculated from an appropriate exposure series for the indicated test conditions. These values are relative and may differ significantly under different salinity or temperature regimes. Appropriate statistical techniques should be used to determine confidence limits when possible.

A preliminary exposure series is helpful in establishing a suitable range of toxicant concentrations. In general, three or four oysters exposed for 48 hours to appropriate concentrations of the test material will bracket the range of toxicant concentrations required to determine 96-hour EC50 data.

REFERENCE

Butler, Philip A. 1965. Reaction of Some Estuarine Mollusks to Environmental Factors. In: Biological Problems In Water Pollution - Third Seminar - 1962. U.S. Department of Health, Education, and Welfare, Public Health Service Publication No. 999-WP-25 June, 1965.

B. CHRONIC BIOASSAY USING SHEEPSHEAD MINNOWS (Cyprinodon variegatus)

1. Purpose and Limitations

The purpose of this procedure is to provide a method of determining the effect of a material on survival of sheepshead minnow embryos and fry, their growth to adulthood, and spawning success. Spawning success is measured by observing the ability of pairs of fish to spawn naturally, their fecundity, fertilization success and survival of embryos and fry.

This test has several limitations and should not be considered valid for assessing toxicity of all types of materials. Sheepshead minnows are tolerant of low oxygen and a wide range of temperature and salinity and bioassays using this fish may underestimate the toxicity of materials that are toxic because they alter these environmental conditions. Materials tested should be ones that mix well with water. Insoluble or highly turbid materials mix poorly, and their toxicity may be under or overestimated.

2. Physical Systems

a. Water Source

1. The source of test water should be (1) from the dump site or (2) a natural source of sea water with salinity ≥15 °/oo.

2. Sea water must be filtered to remove particles 15µ and larger, but filtration should not affect the chemical composition of the natural sea water. Filtration must remove planktonic larvae which could prey upon eggs, fry and juvenile fish in the chronic bioassay.

3. Any source proposed must be analyzed for possible pollutants (e.g., pesticides, PCB's and heavy metals). Special determinations should be made for those toxicants being investigated.

b. Dosing Apparatus

A number of apparatus would be acceptable for this bioassay including those of Mount and Brungs, 1967; Hansen et al. 1971; Hansen et al. 1974 or Schimmel et al. 1974.

c. Toxicant Mixing

A mixing chamber is necessary to assure adequate mixing of the test material. Aeration should not be used for mixing. Mixing is extremely important because if materials are not adequately mixed with water, toxicity can not be properly assessed. Improper mixing can either expose the animal to too much or too little of the material, and toxicity would be over or underestimated.

d. Duplicates

True duplicates should be used for each concentration in all tests (no water connection between aquaria). Aquaria location should be by random selection.

e. Aquaria

Each duplicate glass aquarium should measure a minimum of 30 x 90 x 30 cm high and have a water depth of 15 cm.

f. Embryo and Fry Chambers

1. Embryo and fry chambers should be constructed to allow for adequate exchange of water and to insure that the proper quantity of material is entering the chambers. Embryo chambers (incubation cups) can be constructed from 113 g, 5 cm, OD round glass or beakers with bottoms cut off. The bottoms are replaced with 40 mesh stainless steel or nylon screening. Chambers are hung on an oscillating rocker arm apparatus hat is driven by a 1-5 rpm electric motor (Mount, 1968). These chambers must be brushed daily to prevent clogging. Chambers may also be constructed from Petri dishes to which nylon or stainless steel screen is glued (Schimmel et al. 1974).

2. Embryo and fry chambers should be designed so that water can be drained down to 2.5 cm (1 inch) in order to facilitate growth measurements of fry.

3. Embryo and fry chambers may be supplied test water by (1) separate delivery tubes from the mixing chamber, (2) splitting the flow from the aquaria or (3) egg cups on a "rocker" arm. Care must be taken that each embryo and fry chamber receives an equal amount of the toxicant solution.

g. Flow Rate

1. Flow rates to each duplicate aquaria should be great enough to (1) provide 90% replacement in 8-12 hours (Sprague, 1969); (2) maintain dissolved oxygen, 60% saturation; and (3) maintain the toxicant concentration.

2. The test system should be equipped with an alarm system to insure continuation of water and toxicant flow.

h. Photoperiod

A 16-hour light/8-hour dark cycle should be maintained throughout the test. It may be desirable to control lights by a timing switch (Drummond and Dawson, 1970).

i. Temperature

Test temperature should be maintained at 30°C (±1°C). Temperatures can be maintained by (1) preheating the diluted water to the prescribed temperature, and/or (2) placing the test aquaria in a temperature controlled water bath.

j. Cleaning

All aquaria should be cleaned whenever organic material builds up. Aquaria should be brushed down and siphoned to remove accumulated material. To reduce stress, fish can be left in the aquaria but the end of the siphon should be covered with screen. Care should be exercised in cleaning to prevent loss or damage to the fry, juveniles or adults. Embryo and fry chambers may need to be cleaned frequently or replaced when screens clog or organic material collects. Frequency of cleaning will vary but it may be daily. Special care is required to prevent injury to fry.

k. Spawning Chambers

Chambers should be constructed of either glass or #316 stainless steel. Chambers should be at least 13 x 30 x 15 cm high, with a large mesh screen attached 1 cm above the bottom to enable eggs to pass through. This reduces cannibalism by the parents.

l. Disturbance

All test chambers containing fish should be shielded from excessive outside disturbances. Tanks should be shielded from all outside light sources that would interfere with the photoperiod.

m. Concentrations

1. A minimum of 5 concentrations of toxicant and a control, all duplicated, should be utilized in all acute and chronic tests.

2. Concentrations selected for chronic toxicity experiments should be based on results of acute flow-through bioassays. Concentrations should be selected so that at least one will adversely affect some life stage of the sheepshead minnow and one will not affect any stage.

3. We believe that chemical analyses are justified because of the complexity of this bioassay and the need for interpreting the results. Analyses should be made of the material itself, of the water during this test and of the adult fish at the conclusion of the test. At a minimum,

water from each aquarium at the beginning and end of the test, and fish from each aquarium (10 or more fish each) at the end of the test should be analyzed. It is highly desirable to chemically analyze additional samples of water and of fish including, each life stage, muscle tissue and gametes.

n. Acute Tests

Acute flow-through bioassays should be conducted prior to initiation of any chronic test. It is desirable for these tests to be on at least two different age classes (e.g., fry, juveniles or adults). Consult section on suggested acute flow-through bioassay methods.

3. Biological Systems

a. Source of Adult Fish

Adult fish should be obtained from the same source, either from wild population or suitable culture laboratories. They should be held in flowing 30°C sea water of ≥ 15 $^{o}/oo$ salinity for a minimum of two weeks before obtaining eggs.

b. Obtaining Eggs from Adult Fish

To obtain a sufficient number of eggs to begin a chronic exposure, two methods may be employed: (1) natural spawning from laboratory stocks; and (2) artificial inducement, where egg production is stimulated by injection of human gonadotrophic hormone and fertilized with sperm excised from males. (Schimmel, et al. 1974). The former may be preferable.

c. Beginning the Test

The test begins when fifty fertilized eggs are placed in two or more separate embryo and fry chambers in each duplicate aquarium. Survival of embryos and fry (which constitute the parental stock F_1) are to be checked and recorded daily. After 4 weeks fish are reduced to 25 per duplicate aquarium. This should provide enough fish so that at least five pairs of adults will be available in each replicate aquarium for spawning.

d. Food

1. Fry should be fed live brine shrimp nauplii two or more times daily for about 2 weeks. (Do not use frozen nauplii.) After 2 weeks supplement with dry trout pellets (e.g., Oregon Moist) or dry mollie flakes (e.g., Biorell) for 2 additional weeks.

2. Juveniles and adults can be fed twice daily on dry food supplemented with frozen adult brine shrimp. Each batch of food should be checked for pesticides (DDT, dieldrin, endrin, etc.) and metals. In addition, chemical analysis should also include chemicals in the material to be tested.

e. Disease

Disease outbreaks should be handled according to their nature with each aquarium being treated similarly even though disease is not evident in all aquaria. All treatments should be kept to the minimum and recorded as to type, amount and frequency.

f. Measuring Fish

A sample of fish of the F_1 generation should be measured at one day post-hatch, 4 weeks, 8 weeks and at adult termination. Larval (F_2) fish are to be measured at one day post-hatch and weighed and measured at week 4 (termination). Suggested techniques for measuring fish include the photographic method outlined by McKim, J.M. and D.A. Benoit, (1971) or placing fish in a beaker or petri dish containing .63 cm (1/4 inch) of water on a Xerox machine or other photocopier.

g. Thinning

At 28 days post-hatch, the F_1 larvae should be randomly reduced to 25 fish per duplicate aquaria.

h. Spawning Chambers

As mature adults (F_1) begin courtship (indicated by sexual dimorphism, territoriality, aggressive behavior and courtship by the male), separate pairs should be placed in individual spawning chambers in the aquaria. Pairs should be left in the chambers until a sufficient number of eggs have been collected to insure statistical comparisons of fecundity, fertility, and survival of embryos and fry can be made. A minimum of five pairs of fish should be used in each aquarium for spawning studies.

i. Removing Eggs

1. All eggs should be removed at a fixed time each day so the fish are not overly disturbed and that disruption of spawning activity will not occur.

2. Daily records of spawnings, egg numbers, and egg fertility must be kept. All eggs must be removed daily, examined for fertility, and then used for survival studies or residue analyses, or discarded. Each pair should be observed daily for a minimum of 2 weeks.

j. Egg Incubation

1. Fifty fertile eggs should be collected and incubated from adults in each aquarium. Preferably they should be from each of the 5 spawning pairs. If necessary because of small spawns, eggs can be collected over an extended period in order to obtain 50 eggs.

2. If no spawning occurs in the highest concentration, eggs should be transferred from control spawns and incubated in the highest concentration to gain additional information.

3. Groups of 50 eggs are placed in two or more egg cups. Survival of embryos, time required to hatch, hatching success, and survival of fry for 4 weeks will be determined and recorded.

4. Additional groups of 50 eggs from fish from contaminated aquaria should be placed in control aquaria to determine if they contain chemicals toxic to embryos or fry.

k. F_2 Embryos and Fry

Survival of embryos and fry will be recorded daily for 4 weeks. Fry are to be measured on one day post hatch and weighed and measured on week 4 (termination). Daily records should be kept on mortalities and development of abnormalities.

l. Termination of Adults

1. With many chronic procedures utilizing other fishes, termination of the test is considered the time when no spawning activity has occurred over a 2 week interval. For the sheepshead minnow, however, termination should occur after 2 or more weeks of spawning. Enough spawns have to occur to statistically predict the effect of the material that is tested. It is our experience that most fish will spawn readily and almost daily unless they are affected by a pollutant or are immature.

2. Adult fish should be weighed, measured, sexed and retained for residue analysis.

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C. FISH BRAIN ACETYLCHOLINESTERASE INHIBITION ASSAY

1. Introduction

The purpose of this procedure is to provide a method for determining the effect of materials to be dumped in the ocean on acetylcholinesterase (AChE) in fish brains. This is a test for nerve poisons which disrupt nerve impulse transmission by inhibiting AChE, the enzyme that modulates levels of the neurotransmitter acetylcholine (Koelle, 1963; Karczmar, 1970). This procedure is not necessary for materials that contain no AChE inhibiting poisons.

It has been shown that brain-AChE of fishes is inhibited by *in vivo* exposure to organophosphate and carbamate pesticides under laboratory conditions (Weiss, 1958, 1961; Carter, 1971; Coppage, 1972). Furthermore, environmental water pollution by these pesticides has been monitored by measuring AChE activity in fish brains (Williams and Sova, 1966; Holland et al. 1967; Coppage and Duke, 1971). Coppage (1971) defined the conditions necessary for obtaining reliable and reproducible data in the laboratory AChE assays and reported *in vitro* effects of four pesticides on AChE activity in brains of sheepshead minnows (*Cyprinodon variegatus*). Coppage and Matthews (1974) further refined assay techniques and reported acute effects of *in vivo* exposure to organophosphate pesticides on cholinesterases of four estuarine fishes and a shrimp.

2. Recommended Procedure for Exposing Animals

Fish should be exposed to the material as recommended in the definitive test of the continuous-flow method for acute toxicity using fish and macroinvertebrates as described in this manual. Fish to be assayed for AChE should be from control aquaria and, if possible, from three contaminated aquaria in which some fish have died. Live control fish should be divided into three groups of three to six fish each for assay. Three to six fish from the contaminated aquaria should also be assayed using the following method.

3. Recommended Procedure for AChE Assay

a. Preparation of Fish Brains (3 to 6 brains are pooled for each sample).

1. Weigh 5 cm square of aluminum foil in following manner: pick up and place foil on balance pan with forceps (fingers can leave enough

moisture to cause weight error at this low weight). Weigh, then leave on pan at full rest.

2. Place another larger piece of foil in dissecting area.

3. Kill fish by placing them in a clean beaker containing acetone for about 3 minutes.

4. Pour fish into clean sink or pan and scale heads under running water with scalpel. (Check all dissecting equipment used. Make sure it has been cleaned and rinsed with acetone to avoid pesticide residues. As heads are scaled, place fish in another beaker containing acetone.)

5. Pour fish into clean sink or pan and then place them on paper and blot dry.

6. With scissors, clip away skull from above the brain.

7. After all skulls have been clipped, remove brains by pulling off bone flap with forceps and digging bone and flesh away from spinal cord with probe if necessary. Cut spinal cord about 2 mm behind brain.

8. Strip brain from optic nerves after lifting from behind, and place on larger piece of foil.

9. After all brains are removed, transfer them with forceps to the preweighed foil on the balance pan and determine weight in milligrams. Divide weight by five.

10. Transfer weighed brains to nylon cup (See next section) and add about 4 ml of distilled water.

11. Homogenize for 1 minute then pour into graduated cylinder. Rinse cup several times with distilled water and pour into cylinder.

12. Add distilled water to cylinder until total volume (in ml) equals the number found by dividing the brain weight by five. Pour this into beaker to gently mix. Assay within 30 minutes after preparation.

b. Assay for AChE

AChE activity should be determined by using an automated recording pH stat to measure normal and in vivo-inhibited brain AChE. The following procedure applies: mix 2 ml of diluted brain homogenate with 2 ml of 0.03 M acetylcholine iodide in distilled water; titrate the liberated acetic acid with carbonate-free 0.01 N NaOH; carry out the reaction at pH 7 and 22°C while passing nitrogen over the liquid to prevent adsorption of atmospheric carbon dioxide. Calculate the micromoles of substrate hydrolyzed per unit of time from the number of micromoles of NaOH required to neutralize the liberated acetic acid per unit of time, and express AChE activity as micromoles of ACh hydrolyzed per hour per mg brain tissue in reaction vessel.

4. Calculations and Reporting

Assay results of exposed and control fishes are compared, and percentages of normal brain AChE activity of exposed fish are reported. Results should be subjected to statistical analysis (student's t-test, for example) to determine statistical validity. Original control fish may be divided into groups of five and brains pooled for each group of five to obtain samples for normal AChE and statistical comparisons to exposed fish replicates.

5. Reports

Any deviation from this method must be noted in all reports of results. A report of the results of a test must include:

1. name of method, author, laboratory, and date tests were conducted;

2. a detailed description of the material tested, including its source, date and time of collection, composition, known physical and chemical properties, and variability of the material tested;

3. the source of the salt water, its date and method of preparation;

4. detailed information about the test animals, including name, standard length, weight, age, source, history, and acclimation procedure used;

5. a description of the experimental design, the test containers, the volume of test solution, the number of organisms per concentration, and the loading;

6. period of exposure and number of animals dead at end of exposure;

7. percent of control organisms that died or were affected during the test;

8. methods used for and the results of all test material, dissolved oxygen, pH, and temperature measurements; and

9. any other relevant information.

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16. ABSTRACT

The bioassay procedures given in this manual were developed to provide tests for conducting toxicity evaluations of waste materials considered for ocean disposal under EPA's Ocean Disposal Permit Program.

Nine bioassay procedures are described; three of which are considered "special" and are not recommended for routine use. The procedures specify the use of various organisms representing several trophic levels. Both flow-through and static tests are included. Methods given vary in their utility and complexity of performance. The procedures are not intended to be considered "standard methods", but are to be used as reference methods or official methods dependent on the judgement of the EPA Regional Administrator responsible for the management of the permit program.

17. KEY WORDS AND DOCUMENT ANALYSIS

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